Adenosine signalling in inflammation

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Abstract: A transcription approach for the identification and characterization of downstream effector mechanisms. Veterinary clinical considerations and pharmacologic potential in equine chronic airway disease.

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A transcriptomics approach for the identification and characterization of downstream effector mechanisms

Veterinary clinical considerations and pharmacologic potential in equine chronic airway disease

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Contents

1 Introduction

The first hint suggesting that adenosine may act as an extracellular signalling molecule dates as early as 1929 (Drury and Szent-Gyorgyi, 1929). These authors observed a decrease of heart rate and an increase in coronary blood flow after the injection of heart extracts into animals. The molecule responsible for this effect turned out to be adenosine, and the first therapeutic applications of this nucleoside were based on its negative chronotropic effect on patients with supraventricular tachycardia (diMarco et al., 1985). Beyond its effects on the cardiovascular system, adenosine has been shown to influence the function of almost every organ system that has been studied.

Adenosine homeostasis

Adenosine is a nucleoside composed of an adenine molecule attached to a ribose (ribofuranose) via a glycosidic bond and it is a metabolic by-product of adenosine triphosphate (ATP) degradation during energy generation. Adenosine arises mainly from the cleavage of adenosine monophosphate (AMP) by either intracellular or extracellular 5'-nucleotidases, and efficient equilibrative transporters balance intracellular and extracellular adenosine levels. Hydrolysis of S-adenosyl homocysteine has also been reported to be a minor pathway of adenosine formation (Deussen et al., 1989). Adenosine binds to G-protein coupled receptors and activate various signalling cascades, which ultimately result in multiple biological responses. In physiological conditions, the level of adenosine remains low due to its conversion to AMP by adenosine kinase (Arch and Newsholme, 1978). Adenosine deaminase also contributes to adenosine degradation to inosine and hypoxanthine (Trams and Lauter, 1974). In metabolically stressful conditions such as ischemia, sepsis and hypoxia, extracellular adenosine concentration increases in proportion to energy consumption, ATP degradation and AMP accumulation, reaching local concentrations of up to 30 μM – a 150-fold increase over basal levels (Van Belle et al., 1987). During metabolic stress adenosine kinase is also inhibited, thereby contributing to adenosine accumulation (Fig 1).

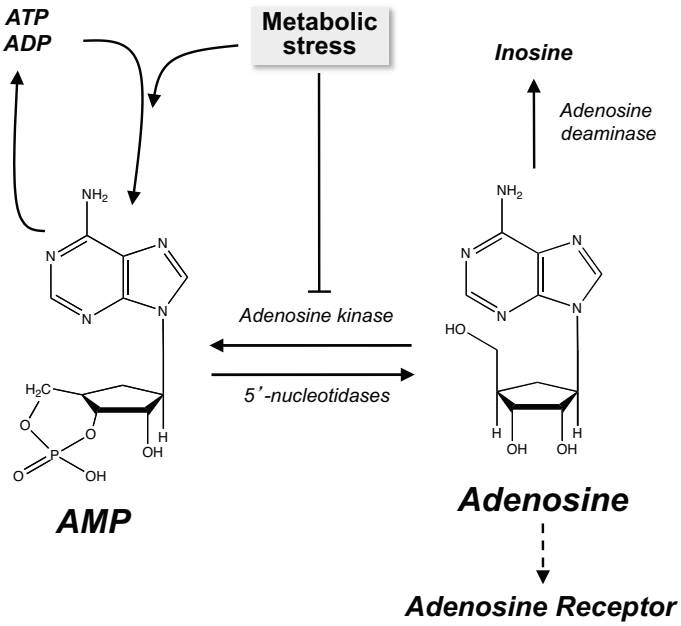

Fig 1- Adenosine metabolism in physiological and pathological conditions: metabolic stress leads to increased ATP and ADP degradation and also inhibits adenosine kinase.

Biological functions of adenosine

Adenosine acts as a chemical messenger with varied functions in different organs systems. In the central nervous system adenosine has been reported to integrate and fine-tune glutamatergic and dopaminergic neurotransmission acting as an inhibitory neuromodulator capable of downregulating central nervous system (CNS) excitability at different levels, and of influencing cerebral blood flow (Shi et al., 2008), basal ganglia function (Schwarzschild et al., 2006), seizure susceptibility (Pagonopoulou et al., 2006), pain perception (Sawynok and Liu, 2003), sleep induction (Basheer et al., 2004) and respiration (Lahiri et al., 2007). The observation of caffeine's excitatory effect on the CNS has stimulated the search for adenosine receptor (AR) antagonists for the treatment of various conditions. Because caffeine and other methylxanthines antagonize the subtype A_1 of AR (at significantly lower concentrations that its effect on phosphodiesterase inhibition), antagonists for this receptor subtype have been tested for the treatment of several conditions, including dementia and anxiety disorders (Maemoto et al., 2004), cerebral ischemia (Ribeiro, 2005), seizures (During and Spencer, 1992), pain, Parkinson's disease (Schapira et al., 2006) and Huntington's disease (Blum et al., 2003).

Adenosine is a potent vasodilator in most vascular beds, with the exception of renal afferent arterioles and hepatic veins. The vasodilating properties of adenosine have long been recognized and this nucleoside is in fact marketed for myocardial

perfusion scintigraphy in humans. Adenosine can also induce angiogenesis through direct and indirect mitogenic effects on vascular cells. The main pro-angiogenic actions of adenosine have been tracked down to its ability to regulate the production of pro- or anti-angiogenic substances from vascular cells through hypoxia-inducible factors (HIFs) such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) within the microenvironment of hypoxic tissues. Adenosine also protects the myocardium (as well as other tissues) from hypoxia and reperfusion injury through ischaemic preconditioning (recently reviewed by Das and Das, 2008). In addition, adenosine shows anthiarrythmic effects (reduction of both heart rate and contractility) and it is intravenously administered to restore normal heart rhythm in patients with paroxysmal supraventricular tachycardia. However, for conditions requiring repeated applications more selective agonists for the A_1 subtype of AR are required to reduce undesirable effects in other organs and systems.

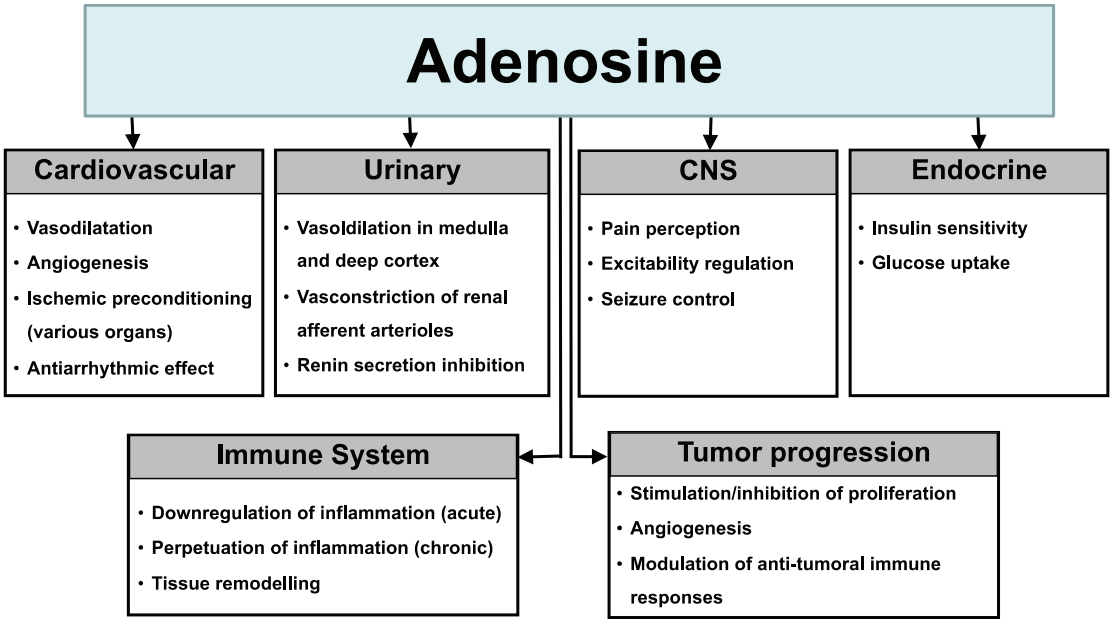

Fig 2- Effects of adenosine in various organs and systems, during inflammation and tumor progression

Extracellular adenosine affects several kidney functions both at the vascular and tubular level. First, this nucleoside can significantly reduce the glomerular filtration rate by activating the A_1AR on afferent arterioles (Hansen and Schnermann, 2003). Second, adenosine-induced vasodilation through the A_{2A}AR in the deep cortex and medulla contributes to minimize medullary injury during hypoxic episodes. Third, AR activation has been shown to inhibit the secretion of renin (Edlund et al., 1994). Fourth, adenosine has been shown to prevent ischemia-reperfusion injury in the kidney though ischemic preconditioning (Lee and Emala, 2000).
Adenosine has also been implicated in glucose metabolism and lipolysis. Activation of A_1AR has been associated with an increase in plasma insulin, increase in insulin

sensitivity and glucose uptake (Dong et al., 2001). Furthermore, A_1AR agonists have been tested in human as adjuvant therapy in obesity-related insulin resistance and type 2 diabetes, and resulted in improved tryacilglycerol levels and reduced fatty acid levels (Donnelly and Qu, 1998). In addition, experimental evidence in a rat model of type 2 diabetes suggests that A_{2B}AR is involved in adenosine-induced glucose production in hepatocytes.

The fact that significant levels of adenosine are found in the hypoxic extracellular milieu of solid tumors have suggested a role for this nucleoside in cancer progression (Blay et al., 1997). In fact, AR activation can inhibit cell growth in human thymocytes (Szondy, 1994) and glomerular mesangial cells (Zhao et al., 2002) through cell cycle arrest or apoptosis, and adenosine has been shown to modulate tumor cell proliferation in human colonic adenocarcinoma (Lelievre et al., 1998), epidermoid carcinoma cells (Tey et al., 1992), human breast cancer cells (Panjehpour and Karami-Tehrani, 2007) and to induce apoptosis in human promyelocytic leukaemia cells (Kohno et al., 1996b). However, this nucleoside can have the opposite effect and was proven to stimulate normal cell proliferation in several cell types including Swiss mouse 3T3 and 3T6 fibroblasts (Rathbone et al., 1992), hematopoietic cells (Fishman et al., 2000) and arterial endothelial cells (Grant et al., 1999). In addition, recent evidence suggests that adenosinergic signalling may suppress and redirect immune responses within the hypoxic tumor environment (reviewed by Sitkovsky, 2009). The transcriptional activities derived from AR activation appear to collaborate with hypoxia inducible factors (HIFs) in stimulating immunomodulation by T-regulatory cells, by upregulating transforming growth factor (TGF)-β, interleukin 10 and Galectin-1, thereby providing cancerous tissues protection from destruction by cytolytic anti-tumor T-cells (Lappas et al., 2005; Sitkovsky and Lukashev, 2005; Zarek et al., 2008). Extracellular adenosine can also directly affect the function of $CD8^+$ T effector cells, which predominantly express A_{2A} and A_{2B}ARs and signalling through these receptors might redirect the repertoire of TCR-induced inflammatory cytokines. A recent report has shown that A_{2A}AR activation promotes peripheral tolerance by inducing T-cell anergy (Zarek et al., 2008). These results support the pioneer findings by Ohta and collaborators, who observed that A_{2A}AR protected tumors from antitumor T-cells (Ohta et al., 2006).

Paradoxical role of adenosine in inflammation

The rapid accumulation of adenosine that takes place during hypoxia, tissue injury and acute inflammation is followed by a number of tissue protective responses. On one hand, extracellular adenosine represents a pre-eminent alarm molecule that reports tissue injury in an autocrine and paracrine manner to surrounding tissue. On the other, it decreases tissue energy demand via a direct inhibitory effect on

parenchymal cell function and provides a more favourable cellular environment by increasing nutrient availability via vasodilatation (Linden, 2001). Adenosine also helps to maintain tissue integrity by modulating the immune system and by regulating exuberant immune responses. Based on these principles, adenosine and AR occupancy on cells of the immune system constitute a potential target for inflammatory pro-resolving therapy. As a matter of fact, a number of drugs currently used are believed to mediate their anti-inflammatory effect, at least in part, by increasing adenosine signalling. These include methotrexate, sulphasalazine and FK506 (Hasko and Cronstein, 2004).

During chronic inflammatory processes, however, the sustained formation of adenosine has been associated with deleterious effects. Elevated adenosine concentrations can be found, for example, in bronchoalveolar lavage (BAL) and exhaled breath condensate of human patients with asthma (Driver et al., 1993; Huszar et al., 2002), where it contributes to airway hyperresponsiveness, inflammation perpetuation and airway remodelling.

These seemingly paradoxical effects can be explained by the activation of alternate ARs on inflammatory cells, which result in divergent signalling cascades and different effector pathways, some of which remain to be discovered.

Table 1- Adenosine's mechanisms in resolution or perpetuation of inflammation

Roles of adenosine in inflammation resolution (acute)	Roles of adenosine in inflammation perpetuation (chronic)
• Prevention of leukocyte trafficking • Reduction of macrophage activation • Downregulation of pro-inflammatory cytokines in several immune cell types • Upregulation of anti-inflammatory IL-10 in macrophages • Reduction of platelet and lymphocyte activation	• Upregulation of pro-inflammatory cytokines (i.e. IL-4, IL-6, IL-8, IL-13, MCP-1) • Fibroblast to myofibrobalst differentiation (pro-fibrotic) • Tissue remodelling (i.e. protease-mediated airway destruction) • Mucus metaplasia

Adenosine receptors and intracellular signalling

Although the bioavailability of adenosine is an important determinant of its biological functions, the pattern of expression and distribution of the ARs in the anatomical–structural sites accounts for the observation that adenosine may exert either deleterious or protective roles. There are 4 distinct AR subtypes known to-

date: A_1, A_{2A}, A_{2B} and A_3. ARs have been cloned in number of species, including dog, sheep, horse and cow, as well as in man. Usually, more than one adenosine receptor is expressed in a single cell resulting in a composite pharmacological response. ARs are densely expressed on virtually all inflammatory cell types, including mast cells (Feoktistov and Biaggioni, 1995), neutrophils (Fortin et al., 2006), eosinophils (Kohno et al., 1996a), monocytes and macrophages (Hasko et al., 2007; Khoa et al., 2006), dendritic cells (Panther et al., 2001), lymphocytes (Apasov et al., 2000), NK and NKT cells (Harish et al., 2003; Lappas et al., 2006), as well as fibroblasts (Chan et al., 2006), platelets (Gessi et al., 2000), endothelial and epithelial cells (Li et al., 1998; Sitaraman et al., 2001).

A_1AR

This subclass of AR has been associated with both pro- and anti-inflammatory effects. Engagement of the A_1AR has been shown to promote activation of human neutrophils and monocytes, thus leading to proinflammatory responses (Merrill et al., 1997; Salmon et al., 1993). In line with this, it has been shown in a rabbit model of allergic asthma that A_1AR activation contributes to bronchoconstriction, suggesting a participation of this AR subtype in acute phases of asthma (Ali et al., 1994). Similarly, A_1AR receptor antagonism has been shown to be beneficial in attenuating ischemia reperfusion and endotoxin-induced lung injury (Neely et al., 1997)

In contrast, the genetic removal of this AR subtype in adenosine deaminase-deficient mice ($ADA^{-/-}$) resulted in enhanced pulmonary inflammation, increased mucus metaplasia and alveolar destruction (Sun et al., 2005), meaning that in a scenario of increased adenosine concentration, A_1AR contributes to inflammation control and tissue protection. This class of AR was also shown to have an anti-inflammatory and a protective role in experimental animal models of injury in the heart, nerves and kidney (Lee et al., 2004; Liao et al., 2003; Tsutsui et al., 2004).

A_{2A}AR

This subtype of ARs is expressed in numerous organ systems throughout the body. Leukocytes, platelets and neurons in certain areas of the central nervous system express the A_{2A}AR abundantly, while intermediate levels have been found in lung and heart (Fredholm et al., 2001). A_{2A}AR have been found co-expressed with A_{2B}AR in human mast cells from bronchoalveolar lavage cells (Feoktistov and Biaggioni, 1998; Suzuki et al., 1998). Several models have shown a strong link between activation of the A_{2A}AR and the down-regulation of inflammation and tissue damage in the lung and other organs (Ohta and Sitkovsky, 2001; Thiel et al., 2005). It has now become clear that engagement of A_{2A}ARs influences different aspects of the inflammatory process, such as neutrophil activation and degranulation, oxidative species production, the expression of adhesion molecules, cytokine release and mast cell degranulation (Lappas et al., 2005). Furthermore, A_{2A}AR agonists have been

proposed to control inflammation and inflammation-related tissue injury. For example, CGS-21680 (a selective A_{2A}AR agonist) has been found to protect the lung of Sprague-Dawley rats against shock-induced injury, suggesting a novel therapeutic approach against organ injury following trauma/hemorrhagic shock (Hasko et al., 2006). This compound has also been studied in a rat model of allergic asthma, in which it produced a broad-spectrum of anti-inflammatory activities (Fozard et al., 2002).

A_{2B}AR

A_{2B}AR is a low-affinity receptor that requires significantly higher concentrations of adenosine to be activated, such as the ones measured during inflammation and hypoxia. In addition to mast cells (Feoktistov and Biaggioni, 1998), human A_{2B}ARs have been found on smooth muscle cells (Zhong et al., 2004), lung fibroblasts (Zhong et al., 2005), endothelial cells (Feoktistov et al., 2002), bronchial epithelium (Clancy et al., 1999). Activation of A_{2B}AR has been unequivocally associated to pro-inflammatory biological events, including vasodilation (Martin, 1992), facilitation of antigen-induced degranulation in human mast-cells (Feoktistov and Biaggioni, 1995) and increased release of interleukin-6 and monocyte chemoattractant protein 1 (MCP-1) from epithelial cells, astrocytes and fibroblasts (Sitaraman et al., 2001; Zhong et al., 2005). Also, A_{2B}AR signalling augments interleukin-8 (IL-8) release and upregulates T-helper 2 (Th2) type cytokines (IL-4 and IL-13) in human mast cells, and promotes IgE synthesis by B-lymphocytes (Ryzhov et al., 2004). Therefore, blockage of A_{2B}AR has been proposed as an anti-inflammatory approach. In support of this concept, $ADA^{-/-}$ mice (which accumulate adenosine) treated with the A_{2B}AR antagonist CVT-6883 showed less pulmonary inflammation, fibrosis, and alveolar airspace enlargement than controls (Sun et al., 2006b).

A_3AR

In human, a relatively high density of functionally active A_3ARs can be found in eosinophils and elevated mRNA levels of this AR have been detected in lung biopsies of patients with asthma and in activated lymphocytes (Gessi et al., 2004). Although the functional role of this AR subtype remained controversial, it has now become clear that at least some of the observed inconsistencies can be explained by interspecies differences. In rats and guinea pigs A_3AR activation can trigger mast cell degranulation (Fozard et al., 1996; Shepherd et al., 1996) and A_3 receptor antagonism attenuates pulmonary inflammation, reduces eosinophil infiltration and decreases airway mucus production (Young et al., 2004). In contrast, specific activation of the A_3AR with the selective agonist IB-MECA in humans inhibits eosinophil chemotaxis and other proinflammatory functions (Walker et al., 1997). Interestingly, this same compound has been able to attenuate the indices of injury and apoptosis following lung reperfusion in an isolated cat lung model and these effects could be blocked by

the administration of MRS1191, a dyhydropyrimidine that antagonizes A_3 AR selectively (Rivo et al., 2004). In addition, IB-MECA can trigger anti-inflammatory effects in experimental animal models of collagen- and adjuvant-induced arthritis. In this context a combined therapy of A_3AR agonist and methotrexate in adjuvant-induced arthritis in rats yielded an additive anti-inflammatory effect (Ochaion et al., 2006). New and more potent A_3AR agonists are currently being developed, some of which are already in clinical trials for the treatment of rheumatoid arthritis (Silverman et al., 2008).

Transcriptional effectors of adenosine signalling

The interactions of each AR subtype with alternate G-coupled proteins dictates which signal transduction pathway will be activated or inhibited. Table 2 summarizes the G-coupling proteins involved in AR signalling and the downstream signalling paths involved.

Most of the best characterized AR effects are mediated by the modulation of the adenylate cyclase activity and intracellular cAMP concentration, which in turn activate downstream signalling pathways such as phospholipase C (PLC), protein kinase A (PKA) and protein kinase C (PKC), phosphoinositide 3 (PI3) kinase and mitogen-activated protein kinases (MAPK) (recently reviewed by Jacobson and Gao, 2006). A_{2A}AR signal mainly by activation of the adenylate cyclase/protein kinase A (PKA) canonical pathway, but they also through the activation of an exchange factor that is directly activated by cAMP (Epac) (Fredholm et al., 2007). In addition, several studies indicate that inhibition of NF-kB activity, a well-characterized pro-inflammatory transcription factor, is the direct target of cAMP-mediated anti-inflammatory effects. Genetic evidence in A_{2A} deficient mice indeed shows that an increase in pro-inflammatory cytokines at the transcriptional level in these animals is associated with enhanced NF-kB activity (Lukashev et al., 2004). In support of this hypothesis, a recent study has shown that A_{2B} activation leads to deneddylation of cullin-1 (Cul-1), which in turn suppresses NF-kB activity (Khoury et al., 2007). On the contrary, some other studies have shown that adenosine can mediate a decrease in TNF-α independently of NF-kB, suggesting that adenosine's immunomodulatory effects depend on other transcriptional factors, at least in the absence of the A_{2A}AR (Nemeth et al., 2003b).

The cAMP responsive element-binding protein (CREB) appears as another key adenosine-modulated transcription factor implicated in the regulation of many inflammatory cytokines. CREB can be activated both by PKA and via a p38 MAPK-mediated mechanism (Nemeth et al., 2003a). Interestingly, phosphorylated CREB has been shown to compete with NF-kB p65 for the rate-limiting factor, CREB-binding protein (CBP) (Lin et al., 1997). CREB may therefore inhibit the transcriptional activity of NF-kB, and subsequently suppress cytokine expression (i.e. TNF) in immune cells (Bshesh et al., 2002).

Table 2 – Coupling of ARs to G-proteins and downstream signalling pathways

AR subtype	G protein	Downstream signalling	Reference
A_1AR	G_i	↓ cAMP	(Londos et al., 1980)
		↑ PLC	(Rogel et al., 2005)
		↓ Ca^{2+} Channels	(Fredholm et al., 2001)
		↑ K^+ Channels	(Belardinelli et al., 1995)
A_{2A}AR	G_s *	↑ cAMP	(Fresco et al., 2004)
		↑ PLC	
		↑ IP_3	(Offermanns and Simon, 1995)
A_{2B}AR	G_s	↑ cAMP	(Brackett and Daly, 1994)
		↑ IP_3	
	G_q	↑ PLC	(Feoktistov and Biaggioni, 1995)
A_3AR	$G_{i\ 1,\ 2,\ 3}$	↓ cAMP	(Zhou et al., 1992)
		↑ Ca^{2+} Channels	(Fossetta et al., 2003)
		↑ PLD	(Mozzicato et al., 2004)
	G_q	↑ PLC	(Gessi et al., 2004).

* G_{olf} in striatum (Kull et al., 2000)

The nuclear factor of activated T-cells (NFAT) is a transcription factor capable of integrating signals originated from A_{2B} coupled to Gq and Gs and transduced by cAMP and IP_3 respectively, in HMC-1 mast cells (Ryzhov et al., 2006). Downstream components of the JNK activation pathway have been reported to be up-regulated in asthma (Demoly et al., 1995) along with enhanced NF-kB activation in both asthma and COPD (Teramoto and Kume, 2001; Yagi et al., 2006). Therefore, inhibition of MAPK and NF-kB appear to be suitable targets for chronic airway inflammatory conditions.

The activity of the dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) is also regulated by adenosine A_{2A} receptors via increase of cAMP production and stimulation of PKA. DARPP-32 phosphorylation, and its subsequent action as a regulator of multiple other phosphoproteins, has been shown to be important for the effects of caffeine (Lindskog et al., 2002), and is probably relevant for other AR binding drugs.

A series of studies has shown the involvement of the extracellular signal regulated kinase (ERK) in adenosine signalling. A_{2A} appears to mediate ischemic preconditioning by ERK activation (Kis et al., 2003); the protective effect of NECA against infarction was completely blocked by ERK specific inhibitors (Yang et al., 2004). Despite this evidence, the downstream targets of ERK after AR activation remain to be determined.

Besides the canonical activation of the adenylate cyclase by the C-terminus of A_{2A}AR, recent studies have shown that the intracellular end of this receptor can mediate G protein-independent actions (Burgueno et al., 2003; Milojevic et al., 2006; Sun et al., 2006a), for example by recruiting β-arrestin via a GRK-2 dependent mechanism (Khoa et al., 2006). Activation of arrestin can lead to novel types of signalling that are not directly related to G protein activation and which generally evolve in a different time frame than classical signalling events (Lefkowitz, 2007).

This complex web of signalling ultimately results in an integrated and characteristic response for each AR subtype, including upregulation of inflammatory mediators (de novo synthesis) as well as the released of pre-formed factors. In addition, recent studies suggest that AR signalling can also influence the fate of involved factors at the post-translational level (Liu et al., 2009).

2 Aims of this work and approaches

Although medicinal chemistry has produced a large number of compounds able to bind the ARs selectively in recent years (recently reviewed by Jacobson, 2009) our understanding about the underlying effector mechanisms involved in adenosine acting as a 'switch' between stimulation and resolution of inflammation has lagged behind. For this reason, the further development of AR-based strategies of immunomodulation requires knowing the exact molecular mechanisms that mediate the inhibition/stimulation of inflammatory processes by adenosine. Interestingly, many observations sustaining the potential of selective AR activation in the treatment of inflammation and inflammation-related diseases originate from studies in experimental animal models; however, the role of adenosine in veterinary medicine has hardly been investigated. The main aim of these studies was, therefore, not only to gain an insight into the molecular basis of adenosine signalling, but also to explore the potential of AR-based therapies in veterinary medicine. As a consequence, in this work two complementary approaches were employed. On one hand, we explored the molecular effectors of adenosine's anti-inflammatory activity through a combination of well-established cellular systems and novel high-throughput technologies (chapters 3 and 4). On the other, we investigated the presence of a functional adenosine signalling axis in the equine airways (chapters 6 and 7), while chapter 5 provides a link between our current understanding of adenosine signalling and veterinary clinical aspects.

The first half of this work deals with the identification and characterization of cellular signals triggered by the activation of alternate ARs employing a pharmacogenomics approach. A central aspect of adenosine's role in inflammation is the interplay between different signalling cascades, which will ultimately determine the shutdown or the perpetuation of inflammation. In this context, our specific aim was to investigate how A_{2A}AR signalling translates into inflammatory downregulation. Biotechnological advances in the last 15 years have delivered powerful methods to scan for biological responses across the whole genome. The completion of the human genome sequencing has been a milestone that translated into a new set of powerful research tools. Transcriptomics is such a technique and involves the simultaneous detection of all genes being transcribed in a cell at any given time. The set of genes that are actively transcribed (the transcriptome) is influenced by cellular incoming signals. For example, cells respond to stimuli such as apoptotic or proliferation signals, genotoxic insults and other types of chemical stress by up- or downregulating the expression of certain genes, thereby generating characteristic expression signatures. In the context of pharmacological research,

transcriptomics strategies (sometimes referred to as pharmacogenomics) are based on the concept that specific changes of gene expression will reveal targets specifically upregulated by the compound under investigation, giving clues about their mechanism/s of action. Based on this concept, we hypothesized that by collectively analysing the transcriptional changes induced by selective AR activation we could identify at least some of the mechanisms mediating adenosine's paradoxical effects on inflammation.

Chapter 3 constitutes a preliminary study that gives an insight into genome-wide transcriptional analysis, its complexity and its advantages. Transcriptomics relies on a complex workflow of sequential procedures. Some of the most critical steps include cell culture, RNA isolation, reverse transcription and amplification of cDNA, hybridization on the microarray and gene expression data acquisition. Importantly, experimental design requires special consideration when planning a transcriptomics study, with reproducibility being a major challenge, requiring careful consideration of the number of replicates (experimental and/or technical replicates). Genome-wide transcriptional analysis results in very large datasets that have to be processed by proper statistical tools (e. g. *t*-test, multiple component analysis, clustering analysis, analysis of variance) and interpreted in the biological context, therefore requiring expertise in data analysis. In our studies, high-density Genechip microarrays (Human Genome U122 plus 2.0 microarrays, Affymetrix) were employed, which allow the simultaneous comparison of around 47'000 transcripts covering the entire human genome. In the analysis phase several data mining tools were employed, including Agilent's GeneSpring GX (Agilent, Palo Alto, CA, USA), The Expressionist (Genedata, Basel, Switzerland) and Metacore (GeneGo, St.Joseph, MI, USA). Despite the particularities of each of software platform, all of them involve sequential analysis steps starting with microarray quality assessment, followed by condensation of probe sets, data normalization to local background (chip) and to endogenous controls, filtering and calculation fold changes between conditions (i.e. pair-wise or multiple groups analysis). In addition, some these bioinformatics tools can assist in the interpretation of the data generated by performing gene ontology analysis and by cross-comparing the profiles obtained with networks built on curated experimental data. Chapter 3 represents a comprehensive example on the technicalities of transcriptomics approaches and also draws on the advantages of using real-time polymerase chain reaction (RT-PCR) as a confirmatory method. The manuscript is centred on a different class of receptors, the estrogen receptors alpha and/or beta, and describes gene expression profiles induced by phytoestrogens through these receptors. It constitutes an example of how transcriptomics can be applied to toxicological research (toxicogenomics) and is included this thesis because it summarizes the preparatory work that led to approach employed for the identification of AR downstream targets (in the following chapter).

In chapter 4 a similar transcriptomics design was employed to comprehensively analyse AR-subtype specific transcriptional targets in human mast cells. Mast cells are a key cellular type in the initiation and maintenance of inflammatory responses. Because of their strategic location at the host-environment interface and because upon activation by intruder pathogens they release a number of mediators that act locally and traffic to distal sites promoting immune cell trafficking (Shelburne et al., 2009), mast cells have been referred to as the 'generals' in innate immune responses, with neutrophils, monocytes and even lymphocytes being the 'soldiers'. Mast-cell derived inflammatory mediators have a leading role in asthma, in chronic bronchitis, and in chronic obstructive pulmonary disease, COPD (Brightling et al., 2003). Here, we employed the human mast cell-line HMC-1, which expresses AR A_{2A}, A_{2B} and A_3, and which show a striking degree of similarity to primary skin mast cells in their repertoire of transcription factors (Nilsson et al., 1994). Based on this, HMC-1 cells have frequently been employed as an in vitro surrogate model to study molecular processes associated to adenosine signalling. Interestingly, this cell line lacks the high-affinity IgE-receptor, FcεRI, and therefore represents a model for IgE-receptor *independent* cellular events and for studies of more general aspects of mast cell biology (Ali et al., 2000; Kandere-Grzybowska et al., 2003). The aim of this study was to investigate how A_{2A}AR signalling translates into inflammatory downregulation. Therefore, we assessed transcriptional reprogramming of HMC-1 cells treated with a non-selective AR agonist (adenosine analogue) and compared these changes with those obtained by selective A_{2A}AR activation (A_{2A}AR agonist) and by those of non-A_{2A}AR mediated responses (adenosine analogue plus an A_{2A}AR antagonist). For every condition fold changes in transcription were compared to baseline values of untreated controls and gene expression profiles compared between the 3 scenarios. The genome-wide transcriptional screening experiments were performed at the Functional Genomics Center Zurich (FGCZ), University of Zurich-Irchel, and revealed a sharp induction of transcriptionally active NR4A2 and NR4A3 by the adenosine analogue NECA. These results were confirmed by RT-PCR and western blot at the protein level. Furthermore, through the combination of reporter assays and pharmacological inhibition of signalling kinases we established a previously unknown link between AR activation and nuclear orphan receptors 4A2 and 3, and that A_{2A}AR is able to modulate this pro-inflammatory signalling axis.

In the second part of this work the focus of our investigations was shifted to the potential of AR-based strategies in veterinary medicine. Chapter 5 gives an update of AR signalling at the cellular and molecular level, summarizing AR ligands whose pharmacological characteristics have been tested in animal studies. In this manuscript, the disease areas in which AR receptor ligands may have direct applications are addressed, including endotoxemia, ischemia-reperfusion injury, arthritis, renal, cardiac and CNS pathologies. Special emphasis on the biological

effects of adenosine in pulmonary disorders is placed. In chapters 6 and 7 the role of adenosine as an inflammatory mediator in recurrent airway obstruction (RAO) in horses is investigated. Equine RAO (previously known as COPD) is a common disease of horses that shares some features of human asthma, including chronic lower airway inflammation, reversible airflow obstruction and bronchial hyper-responsiveness. The prevalence of RAO can be as high as 50%, ranging from sub-clinical to overtly manifested cases. Based on the potential of AR modulation in chronic airway disease, we reasoned that ARs could prove to be relevant pharmacological targets in this disease. To prove this point, 2 complementary approaches were used.

First, in chapter 6 we analysed bronchoalveolar lavage fluid samples for adenosine content. Samples were obtained both from horses diagnosed with RAO and from healthy individuals presented at the Equine Clinic at the University of Zurich. Upon sample collection and preparation, adenosine concentration was assessed by a specific spectrometry-based assay in collaboration with the Center for Clinical Pharmacology, University of Pittsburgh School of Medicine. These measurements were then compared with the cytological characteristics of the BAL samples and with clinical signs indicative of airway inflammation, such as elevations in respiratory rate, the presence of nasal discharge and nasal flaring, coughing, abnormal tracheal and bronchial sounds or the presence of crackles and wheezes (all of which were combined in a clinical score).

To build on this concept, we next aimed at establishing the effects of AR signalling in the equine airways. The study in chapter 7 addressed 2 main questions. First, the expression of the 4 AR subtypes in BAL cells was established by quantitative real-time polymerase chain reaction (RT-PCR) and compared to that in peripheral blood mononuclear cells (PBMCs), cerebellum, cortex, myocardium, aorta, spleen, jejunum, caecum, kidney, adrenal gland, eye and retroperitoneal fat. Based on this information, the second question aimed at assessing the reactivity BAL cells to AR-ligands is evaluated. Because the goal was not only to test AR pro-inflammatory signalling but also to determine the presence of functional A_{2A}AR, the cells were exposed to alternative AR ligands. Upon lavage, BAL cells were cultured and treated with either a non-selective AR agonist (adenosine analogue), a selective A_{2A}AR agonist or by the combination of an adenosine analogue and an A_{2A}AR antagonist *ex vivo*. Since several studies support the role of IL-6 both in equine pulmonary disease and in adenosine signalling in lower airway inflammation, we selected this cytokine as a read-out of pro-inflammatory signalling. Although variations in cytokine levels were not assessed, we identified significant changes in IL-6 expression (mRNA) induced by non-selective AR activation. Interestingly, IL-6 upregulation could be further increased by concomitantly blocking A_{2A}AR, implying that this receptor subtype is present in BAL cells and that its stimulation can lead to a moderation of proinflammatory signalling.

3 Global gene expression profiles induced by phytoestrogens in human breast cancer cells

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Contribution of Ramiro Dip

Co-design and performance of experimental work; data analysis; co-wrote the manuscript

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Global gene expression profiles induced by phytoestrogens in human breast cancer cells

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Abstract

The nutritional intake of phytoestrogens seems to reduce the risk of breast cancer or other neoplastic diseases. However, these epidemiological findings remain controversial because low doses of phytoestrogens, achievable through soy-rich diets, stimulate the proliferation of estrogen-sensitive tumor cells. The question of whether such phytochemicals prevent cancer or rather pose additional health hazards prompted us to examine global gene expression programs induced by a typical soy product. After extraction from soymilk, phytoestrogens were deconjugated and processed through reverse- and normal-phase cartridges. The resulting mixture was used to treat human target cells that represent a common model system for mammary tumorigenesis. Analysis of mRNA on high-density microarrays revealed that soy phytoestrogens induce a genomic fingerprint that is indistinguishable from the transcriptional effects of the endogenous hormone 17β-estradiol. Highly congruent responses were also observed by comparing the physiologic estradiol with daidzein, coumestrol, enterolactone, or resveratrol, each representing distinct phytoestrogen structures. More diverging transcriptional profiles were generated when an inducible promoter was used to reconstitute the expression of estrogen receptor β (ERβ). Therefore, phytoestrogens appear to mitigate estrogenic signaling in the presence of both ER subtypes but, in late-stage cancer cells lacking ERβ, these phytochemicals contribute to a tumor-promoting transcriptional signature.

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Introduction

Epidemiological studies have linked an increased risk of developing mammary or endometrial malignancies to prolonged estrogen exposure due to early menarche, oral contraceptives, nulliparity, late first-term pregnancy, delayed menopause, or an estrogen replacement therapy (McPherson *et al*. 2000, Clemons & Gross 2001, Nelson *et al*. 2002). In contrast, the dietary intake of soy isoflavones such as genistein and daidzein correlates with a lower incidence of breast and prostate cancers (Ingram *et al*. 1997, Branca & Lorenzetti 2005). However, why endogenous estrogen hormones or synthetic xenoestrogens increase cancer risk, whereas natural phytoestrogens appear to exert an opposite preventive action, is not understood.

In the normal resting mammary gland, estrogen receptors (ERs) are expressed in only a small proportion of epithelial cells that are largely non-dividing (Ali & Coombes 2002). In contrast, enhanced expression of ERs is a critical event in the pathogenesis of a majority (∼70%) of breast cancers and, accordingly, the growth of malignant mammary tumors is estrogen dependent in most cases (Hayashi *et al*. 1997, Gruvberger *et al*. 2001, Rice & Whitehead 2006). Like other ER agonists, phytoestrogens stimulate the proliferation of estrogen-sensitive tumor cells in various experimental systems (Hsie *et al*. 1998, Allred *et al*. 2001), and this growth-promoting activity has raised concerns that soy products, or similar phytochemicals, may represent an additional health hazard for vulnerable risk groups (Messina *et al*. 2006). More skepticism regarding the true benefits of

phytoestrogens came from the observation that hyperplasia of the epithelium and other markers of cell proliferation are detectable in breast biopsies of pre- and postmenopausal women after a period of dietary soy supplementation (Petrakis *et al*. 1996, McMichael-Phillips *et al*. 1998, Hargreaves *et al*. 1999). Thus, establishing the consequences of soy intake in populations at high risk for breast or prostate cancer is an important public health issue (Messina *et al*. 2006).

The present study was instigated by the notion that essentially all cellular responses to estrogenic stimuli culminate in transcriptional regulation, even though some of the known effects are considered indirect or non-genomic (Duan *et al*. 2001, McDonnell & Norris 2002, Levin 2003). Thus, oligonucleotide-based microarrays, which are able to detect global expression profiles at the transcriptional level, provide a convenient molecular approach to analyze biological endpoints of phytoestrogen exposure. Particular attention has been given to the question of whether the native hormone 17β-estradiol and phytoestrogens induce similar or different transactivation functions. For example, a gene expression survey performed on immature mice treated with genistein or 17β-estradiol yielded identical transcriptional responses in the uterus (Moggs *et al*. 2004). Other reports concluded that there are significant differences between the transcriptional profiles elicited by genistein or other estrogenic compounds in the reproductive tract of female rats (Naciff *et al*. 2002, Hong *et al*. 2006). Additional analyses were carried out under more tightly controlled cell culture conditions. One of these studies concluded that there is a partial overlap between the expression patterns elicited by 17β-estradiol and phytoestrogens, including genistein, daidzein, and coumestrol, in human MCF7 breast cancer cells (Ise *et al*. 2005). Another contrasting study with MCF7 cells yielded no substantial relationship between the transcriptional effects of genistein and the endogenous hormone 17β-estradiol (Wang *et al*. 2004).

These conflicting results prompted us to examine genome-wide expression profiles in human MCF7 and T47D breast cancer cells exposed to either single phytoestrogens or the natural phytoestrogen mixture extracted from a representative soy product. Soymilk has been selected for this study because it represents a widespread nutritional supplement and it is increasingly used as a basic component of food products for newborns. As a reference control, an analogous extract from cow milk has been included in view of its much lower phytoestrogen content. The comparison of expression data comprising 47 400 human transcripts revealed that the particular fingerprint induced by soymilk phytoestrogens coincides with the known transcriptional response of breast cancer cells to the endogenous 17β-estradiol hormone. In addition, we found that soy phytoestrogens induce nearly identical expression fingerprints as other structurally distinct phytoestrogens from different sources. A phytoestrogen signature that deviates from the characteristic transcriptional fingerprint of 17β-estradiol was observed when an inducible genetic construct was used to express, in addition to ERα, also the ERβ subtype.

Materials and methods

Chemicals

Daidzein and resveratrol were purchased from Sigma–Aldrich; 17β-estradiol, coumestrol, and enterolactone were from Fluka (Buchs, Switzerland). The inhibitor ICI 182 780 was purchased from TOCRIS Bioscience (Avonmouth, UK). All solvents and reagents were of analytical grade quality.

Extraction and analysis of milk samples

The sample preparation procedure developed for extracting phytoestrogens from milk, including the removal of endogenous estrogen hormones, is fully described elsewhere (Antignac *et al*. manuscript in preparation). Briefly, the samples (cow milk or soymilk obtained from a local retailer) were extracted in 10 ml aliquots with acetate buffer (2 M, pH 5.2) and acetone. After centrifugation, the supernatants were collected and the acetone phase was evaporated under a nitrogen stream. Subsequently, an enzymatic hydrolysis was carried out by overnight incubation (52 °C) with a purified *Helix pomatia* preparation (Sigma). This deconjugation step was followed by purification through two successive cartridges combining a reverse (C_{18}) and a normal (silica) stationary phase (SDS, Peypin, France). After evaporation of the methanol eluates, the remaining residues were reconstituted in 30 µl dimethyl sulfoxide (DMSO) for cell culture experiments. Separate cow milk and soymilk samples were subjected to the same procedure, except that a deuterated compound (daidzein-d_3) was included as internal standard. These samples were analyzed by liquid chromatography–tandem mass spectrometry for the quantitative determination of phytoestrogens (Antignac *et al*. 2003). The 17β-estradiol measurements were performed according to a previously described method (Courant *et al*. 2007).

Cell culture and treatments

Human T47D.Luc cells (BioDetection Systems, Amsterdam, The Netherlands) were maintained in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 medium supplemented with sodium bicarbonate, 1 mM L-glutamine, and 7.5% fetal bovine serum (FBS; Invitrogen). The T47Dβ derivatives (Ström *et al*. 2004) were grown in the presence of 1 μg/ml tetracycline. This antibiotic was removed for a period of 48 h to induce the expression of ERβ. The MCF7 cell line subtype BUS (Soto *et al*. 1995) was maintained in DMEM supplemented with 10% FBS. The other antibiotics used were 0.1 U/ml penicillin and 0.1 μg/ml streptomycin (Invitrogen). All cell lines were cultured at 37 °C in xenoestrogen-free plastic (Corning Inc., Grand Island, New York, USA) under humidified air containing 5% CO_2. Before each experiment, the cells were transferred to phenol red-free medium and cultured for 48 h in the presence of 5% dextran/charcoal-stripped FBS (DCC-FBS). DMSO stocks of each test compound were added to the culture medium. Unless otherwise indicated, the final solvent concentration was adjusted to 0.1% (v/v).

Cytotoxicity assays

A commercial kit was used to measure intracellular ATP levels. Briefly, MCF7 cells were grown in multi-well plates as outlined before and exposed to the indicated fractions of soymilk or cow milk extracts. After 24 h, the CellTiter-Glo reagent (Promega) was added and the luminescent signal was recorded in a microplate reader following the manufacturer's instructions. Additionally, the CellTiter 96 and CytoTox 96 assays (Promega) were used to monitor the overall metabolic activity and the release of lactate dehydrogenase.

ER-CALUX assay

The ER-mediated chemical-activated luciferase expression (ER-CALUX) assay was carried out following the instructions provided by BioDetection Systems. Briefly, T47D.Luc cells were seeded in microtiter plates at a density of 5000 cells per well in 0.1 ml phenol red-free medium containing 5% DCC-FBS. After 24 h, the medium was renewed and the cells were incubated for another 24 h followed by the addition of the indicated test compounds dissolved in DMSO. Solvent controls and a standard 17β-estradiol dose response were included on each plate. After 24 h exposure times, cells were harvested, lysed, and assayed for luciferase activity on a Dynex microplate luminometer (Legler *et al*. 1999).

Microarray hybridization, data acquisition, and analysis

After a 24 h treatment with test compounds, cells were collected by trypsinization and total RNA was extracted using the RNeasy kit (Qiagen). Amount and quality of the RNA fractions were evaluated by u.v. spectrophotometry (260 and 280 nm wavelength) followed by examination of the probes by capillary electrophoresis on Agilent Bioanalyzers. The GeneChip expression and IVT (in vitro transcription) labeling kits (Affymetrix) were used for the synthesis of cDNA and complementary RNA respectively. The biotin-labeled RNA was fragmented and hybridized on human genome U133 plus 2.0 microarrays (Affymetrix, Santa Clara, CA, USA) following the manufacturer's instructions. After hybridization (16 h), the microarrays were processed by automated washing on the Affymetrix Fluidics Station 400. Staining of the hybridized probes was performed with fluorescent streptavidin–phycoerythrin conjugates (1 mg/ml; Invitrogen). The scanning of DNA microarrays was carried out on an Affymetrix laser instrument. Microarray quality assessment, condensing of the probe sets, data normalization, and filtering were conducted using the Expressionist software (Genedata AG, Basel, Switzerland). The *t*-tests were performed between controls and treated cells to assess the statistical significance of differentially expressed genes. False discovery rates were determined according to the Benjamini–Hochberg method (Benjamini & Hochberg 1995). Finally, the means of three to five replicates were imported into a Microsoft Excel file for graphical representation and determination of correlation coefficients. The 'Gene Ontology' database (www.geneontology.org) was consulted for the molecular function of each transcript and, for simplicity, only gene products with a known or inferred function are displayed in the figures.

Real-time RT-PCR

PCR quantifications were carried out to validate the microarray hybridization results. Primers for the selected transcripts were obtained from Applied Biosystems (Foster City, CA, USA). Briefly, 100 ng cDNA were mixed with 100 nM forward and reverse primers in a final volume of 25 μl. The reactions were performed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) for 45 cycles (95 °C for 15 s, 60 °C for 1 min) after an initial 10-min incubation at 95 °C. The fold change in the expression of each gene was

calculated using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen 2001), with the abundant glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript as an endogenous control.

Results

Sample preparation and analysis

After liquid extraction and enzymatic deconjugation, the soymilk and cow milk samples were subjected to a two-step fractionation procedure using reverse-phase (C_{18}) and normal-phase (SiOH) cartridges. The quantitative analysis by liquid chromatography coupled to tandem mass spectrometry confirmed that soymilk contains large quantities of the isoflavones genistein and daidzein, whereas cow milk is characterized by the presence of low levels of enterolactone together with trace amounts of other phytoestrogens (Table 1). An additional determination by gas chromatography–mass spectrometry was used to verify that endogenous estrogen hormones, including 17β-estradiol, were removed from the cow milk sample during the final solid-phase extraction step (data not shown). For the subsequence cell culture experiments, each isolate from a 10 ml sample was reconstituted in 30 µl DMSO, which proved to be compatible with the solubility properties of the various phytoestrogens.

Dose-dependent transactivation from a minimal estrogen-responsive promoter

Initially, human MCF7 and T47D breast cancer cells were exposed to increasing concentrations of the soymilk and cow milk extracts. Cell viability was tested 24 h later by measuring the intracellular ATP pool, which is used as an indicator of metabolic activity. The resulting dose responses demonstrated that no cytotoxic reactions were triggered when the cell culture medium contained up to 0.5% (v/v) milk extracts dissolved in DMSO. This lack of cytotoxicity was confirmed when the metabolic activity was assessed by measuring the capacity to reduce a tetrazolium reagent or when the cell integrity was measured by monitoring the release of lactate dehydrogenase (data not shown).

Table 1 Concentrations (ng/ml) determined for the target phytoestrogens in the analyzed soymilk and cow milk samples

	Concentration	
Compound	**Soymilk**	**Cow milk**
Daidzein	1233	8.3
Genistein	5175	5.6
Equol	nd	2.6
Formononetin	9.6	1.8
Biochanin A	nd	nd
Glycitein	98.5	nd
Enterolactone	1.1	5.2
Matairesinol	nd	nd
Coumestrol	nd	nd
Resveratrol	nd	nd

nd, not detected at the limit of detection, which varied from 0.05 to 0.7 ng/ml depending on the compound.

Next, the soymilk and cow milk extracts were tested for their overall estrogenic activity using a standard reporter gene assay. For that purpose, we exploited a stably transfected carcinoma cell line (T47D.Luc) that carries a chromosomally integrated reporter gene sequence (Legler *et al.* 1999). In this genetically modified cell line, the firefly (*Photinus*) luciferase gene is under transcriptional control of a minimal promoter displaying tandem repeats of estrogen response elements (5′-GGTCACTGTGACC-3′). This artificial construct drives the expression of firefly luciferase in response to ER activation. Thus, to monitor estrogenic activity, cell lysates were examined for luciferase activity after a 24 h treatment with progressively increasing concentrations of 17β-estradiol or the different extracts added to the cell culture medium. In all these treatments, the final concentration of the DMSO solvent was 0.1% (v/v).

In a series of control reactions, the synthetic promoter mediated a dose-dependent luciferase induction in response to the 17β-estradiol standard. This expected estrogenic effect reached peak levels at a hormone concentration of 60 pM (Fig. 1). A similar level of reporter gene induction was observed in the cells incubated with soymilk extract. In agreement with its limited phytoestrogen content, however, the corresponding cow milk isolate resulted in only minor reporter gene induction when compared with the soy product (Fig. 1). These responses to the treatment with 17β-estradiol or soymilk extract were completely suppressed by the addition of the ER antagonist ICI 182 780 at a concentration of 0.1 µM (data not shown).

Global expression profiles elicited by natural phytoestrogen mixtures

Previously, we found that the MCF7 breast cancer cell line is markedly more responsive than T47D cells to estrogenic stimuli, thus yielding a wider range of estrogen-regulated genes as well as larger amplitudes of expression changes (Buterin *et al.* 2006). As a consequence, the MCF7 cells were used herein to perform genome-wide analyses of endogenous

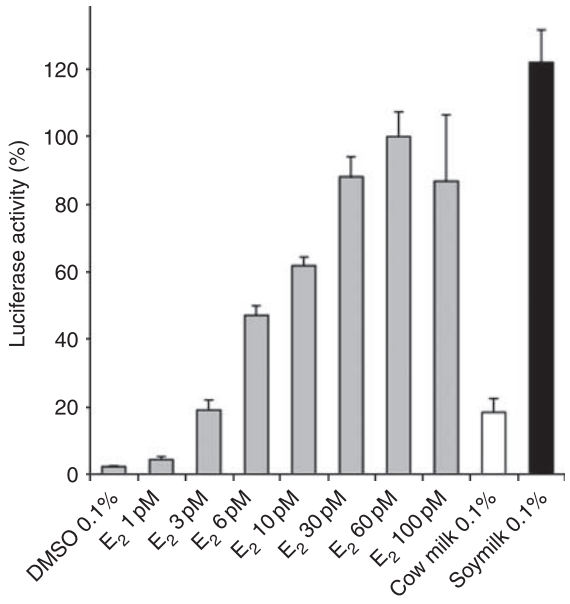

Figure 1 Luciferase reporter gene assay. Stably transfected T47D.luc cells were incubated with 17β-estradiol (E_2 at concentrations of 1–100 pM) as well as soymilk or cow milk extracts. The final concentration of the DMSO solvent was 0.1% (v/v). ER activation was determined by measuring the luciferase induction from a minimal promoter containing repeats of estrogen response elements (mean values of five to six independent experiments). The results are shown in percentage of the induction observed with 60 pM 17β-estradiol.

transcripts after a treatment with phytoestrogen mixtures reconstituted in culture medium. The target cells were incubated in triplicates with soymilk or cow milk extracts to reach a final solvent concentration of 0.1% (v/v). After 24 h exposures, a fraction of RNA from each sample was analyzed using Affymetrix microarrays that display the sequences of 47 400 human transcripts. To identify genes that are susceptible to ER regulation, these microarray data were normalized and subjected to statistical analysis. Also, in view of the large number of regulated genes, the expression data were subjected to a filter for transcripts that exhibited at least a fivefold change relative to the solvent control, thereby eliminating the vast majority of gene products that are more moderately affected or not altered at all following the phytoestrogen treatment. The significance threshold was $P<0.01$, yielding false discovery rates in the range of 0.02–0.1 (Benjamini & Hochberg 1995). According to these criteria, a total of 358 different transcripts were up- or down-regulated by more than fivefold in response to incubation with the soymilk extract. In contrast, the gene expression profile induced by the cow milk sample deviated only marginally from the background transcriptional pattern observed in the solvent control group. In this case, only six transcripts were affected by more than fivefold changes, thus reflecting the much lower phytoestrogen concentration in cow milk.

Figure 2A shows the transcripts displaying the highest amplitude of regulation in response to the treatment with soymilk phytoestrogens. The majority of these transcripts encode for proteins involved in DNA metabolism (ribonucleotide reductase M2 (RRM2), thymidylate synthetase, thymidine kinase 1; see legend to Fig. 2 for abbreviations), DNA replication or recombination (minichromosome maintenance-deficient 10, CDT1, topoisomerase II-α (TOP2A), primase 1, RAD51), cell division cycle (cyclin A2, cell division cycle 2 (CDC2), CDC6, TTK), chromosome segregation and centromere function (aurora kinase B, kinesin-like 7 (KNSL7), Spc24, Spc25, cell division cycle associated 1, kinetochore associated 2, kinesin family member 2C, extra-spindle poles like 1, abnormal spindle-like microcephaly), or inhibition of apoptosis (baculoviral IAP repeat-containing 5). Together with the over-expressed proto-oncogenes myeloblastosis oncogene-like 1 (MYBL1) and MYBL2 as well as two different proliferation markers (antigen identified by monoclonal antibody Ki-67 and Opa-interacting protein 5), this transcriptional profile reflects the typical mitotic signature observed in estrogen-stimulated breast cancer cells (Lobenhofer *et al*. 2002, Coser *et al*. 2003, Frasor *et al*. 2003, Vendrell *et al*. 2004, Buterin *et al*. 2006, Lavigne *et al*. 2007).

A side-by-side comparison indicated that the observed expression changes resulting from exposure to the soy phytoestrogens are similar to the transcriptional effects of the endogenous hormone 17β-estradiol. In fact, all transcripts that were increased by incubation with the soymilk extract were also up-regulated following the treatment with 17β-estradiol (Fig. 2A). To analyze this presumed relationship in more quantitative terms, the mRNA profile induced by the soymilk extract was plotted against the corresponding values obtained with 17β-estradiol. All transcripts were included in this analysis that showed at least a fivefold up- or down-regulation in the phytoestrogen treatment group. The threshold for statistical significance was $P<0.01$. Figure 2B shows that the data points in this comparison grouped in two distinct clusters reflecting those genes that were over-expressed and those that were under-expressed relative to the solvent control. A linear regression analysis of all 358 pairs of data yielded an overall correlation coefficient of $R=0.87$, thereby exceeding the values ($R\approx0.6$) found in another similar study that

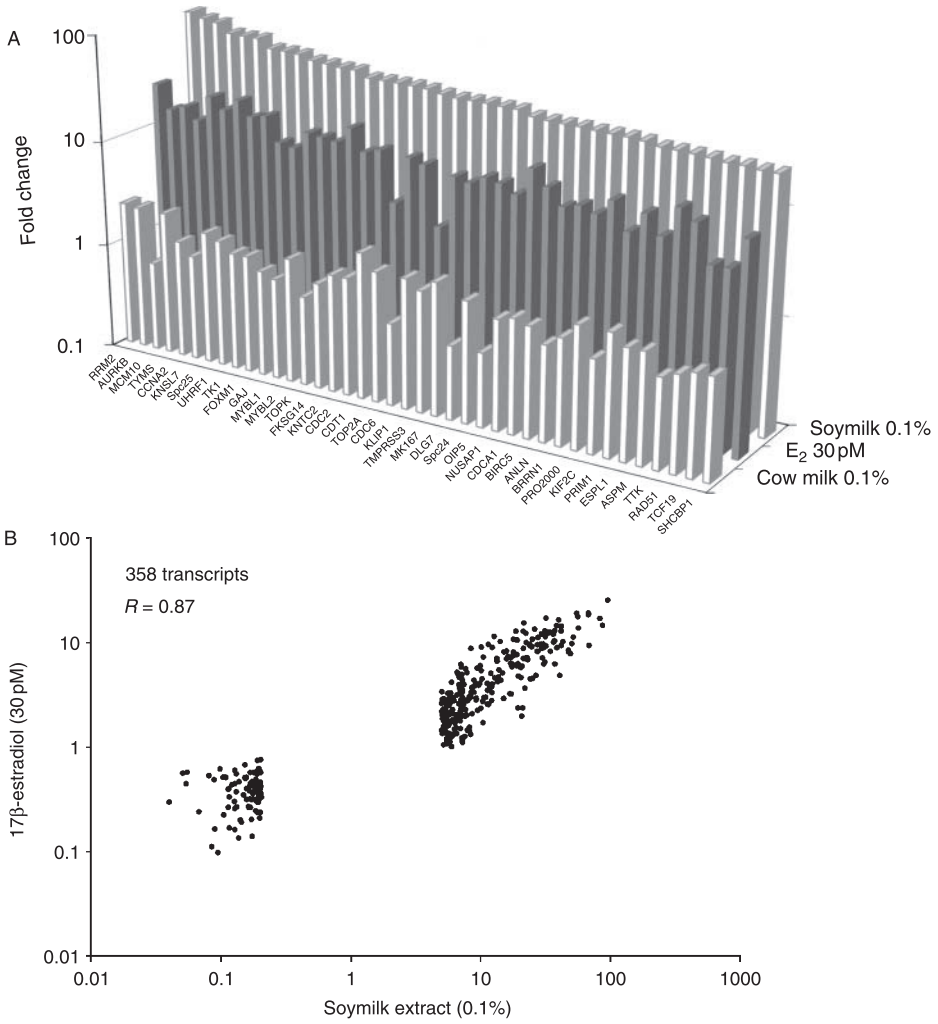

Figure 2 Comparison of genome-wide transcriptional changes in MCF7 cells exposed to milk extracts or 17β-estradiol. (A) Transcripts with the highest amplitude of induction following a treatment with soymilk extract. The level of the same transcripts has also been determined for the treatment with 17β-estradiol (30 pM) or an analogous cow milk extract. The extracts were dissolved in DMSO and added to the cell culture medium to a final solvent concentration of 0.1%. In each case, the fold changes were calculated against the corresponding values obtained in control experiments with solvent alone (mean values of three independent experiments). (B) Linear regression analysis of pairs of data obtained from the experiments with soymilk extract and 17β-estradiol (30 pM). A fivefold change (up- or down-regulation) in the soymilk-treated samples relative to the controls was used as the cut-off to filter the data. A total of 358 transcripts passed the statistical threshold of $P<0.01$. RRM2, ribonucleotide reductase M2; AURKB, aurora kinase B; MCM10, minichromosome maintenance-deficient 10; TYMS, thymidylate synthetase; CCNA2, cyclin A2; KNSL7, kinesin-like 7; Spc25, kinetochore protein Spc25; UHRF1, ubiquitin-like, containing PHD and RING finger domains 1; TK1, thymidine kinase 1; FOXM1, forkhead box 1; GAJ, GAJ protein; MYBL1, myeloblastosis oncogene-like 1; MYBL2, myeloblastosis oncogene-like 2; TOPK, T-LAK cell-originated protein kinase; FKSG14, leucine zipper protein FKSG14; KNTC2, kinetochore associated 2; CDC2, cell division cycle 2; CDT1, chromatin licensing and DNA replication factor 1; TOP2A, topoisomerase II-α; CDC6, cell division cycle 6; KLIP1, KSHV latent nuclear antigen interacting protein 1; TMPRSS3, transmembrane protease serine 3; MKI67, antigen identified by monoclonal antibody Ki-67; DLG7, discs large homolog 7; Spc24, kinetochore protein Spc24; OIP5, Opa-interacting protein 5; NUSAP1, nucleolar and spindle associated protein 1; CDCA1, cell division cycle associated 1; BIRC5, baculoviral IAP repeat-containing 5; ANLN, anilin; BRRN1, barren homolog; PRO2000, PRO2000 protein; KIF2C, kinesin family member 2C; PRIM1, primase 1; ESPL1, extra-spindle poles like 1; ASPM, abnormal spindle-like microcephaly; TTK, TTK protein kinase; RAD51, radiation sensitive 51; TCF19, transcription factor 19; SHCBP1, ortholog of mouse Shc SH2-domain binding protein 1.

compared soy extracts with 17β-estradiol (Ise *et al.* 2005). The close correspondence of expression values, demonstrated in Fig. 2B, lends support to the notion that soy phytoestrogens and 17β-estradiol induce nearly identical transcriptional responses in MCF7 cells. Thus, in contrast to previous reports (Wang *et al.* 2004, Ise *et al.* 2005), we found that the transcriptional machinery of this breast cancer cell line responds in a very monotonous manner to distinct estrogenic stimuli.

Validation using real-time PCR

Real-time reverse transcriptase-PCR (RT-PCR) assays were carried out on representative sequences to confirm the tight correlation between the expression profiles induced by soy phytoestrogens and 17β-estradiol. The following transcripts were tested: RRM2 polypeptide, CDC2, TTK (a protein kinase), and UDP-*N*-acetylglucosaminyltransferase 5. The exposure with soy phytoestrogens was performed with three different proportions of extract in the cell culture medium between 0.001 and 0.1% (v/v). In view of the phytoestrogen contents listed in Table 1, these residue levels translate to genistein concentrations ranging from 0.1 to 10 μM. Incubations with the 17β-estradiol reference were performed using the standard concentration of 30 pM. After normalization with the constitutive GAPDH transcript, expression values are indicated as the ratio between treated cells and solvent controls. A linear regression analysis of the resulting RT-PCR values yielded correlation coefficients of $R=0.92$–0.98, thus confirming that phytoestrogens and the physiologic estradiol hormone induce very similar transcriptional responses (Fig. 3).

Expression patterns induced by single phytoestrogens

We already demonstrated that the most abundant soy phytoestrogen, i.e., genistein, induces global gene expression profiles in both MCF7 and T47D cells

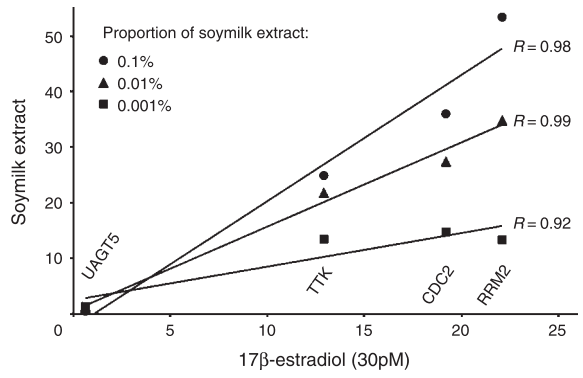

Figure 3 RT-PCR analysis of selected transcripts that were regulated following exposure of MCF7 cells to estrogenic stimuli. The results obtained with three different concentrations of the soymilk extract (0.001–0.1%; v/v) were plotted against the corresponding values obtained in the experiment with 17β-estradiol (30 pM). The fold changes were measured using the GAPDH transcript as an endogenous control (mean values of four to five independent determinations). The linear regression analysis to determine the degree of similarity between the transcriptional changes induced by soymilk extract and 17β-estradiol yielded correlation coefficients of $R=0.92$–0.98.

that are indistinguishable from the transcriptional changes resulting from 17β-estradiol treatments (Buterin *et al*. 2006). A similar convergence was now obtained when we assessed the response of MCF7 cells to another major soy phytoestrogen, i.e., daidzein tested at a concentration of 1 μM (Fig. 4A). Subsequently, this study was extended to different categories of phytoestrogens including coumestrol (found in white clover), resveratrol (found in grape skins and red wine), and enterolactone (an endogenous metabolite generated from plant lignans). The criteria for inclusion of the transcripts into the correlation analyses of Fig. 4 were again a fold change >5 and a corresponding P value <0.01. Compared with the daidzein treatment, the number of significantly regulated transcripts was slightly higher in the coumestrol experiment (Fig. 4B) and markedly reduced in the resveratrol and enterolactone treatments (Fig. 4C and D). However, a direct comparison with the effects of 17β-estradiol on the same human genes yielded correlation coefficients of $R=0.85$–0.92, further supporting the idea that, at least in a low-dose range, all kinds of phytoestrogens generate expression profiles in human breast cancer cells that are superimposable with the transcriptional pattern elicited by a 17β-estradiol stimulus.

Modulation of expression fingerprints by ERβ

RT-PCR quantifications with oligonucleotide primers specific for each ER subtype showed that the predominant mRNA in MCF7 and T47D cells is the one coding for ERα, whereas ERβ transcripts remained undetectable (data not shown). These findings are consistent with previous studies reporting the presence of only trace amounts of ERβ transcripts in T47D and essentially no such transcripts in MCF7 cells (Legler *et al*. 1999, Lobenhofer *et al*. 2002). By immunoblotting methods, it has also been shown that there is no detectable ERβ protein in T47D cells (Ström *et al*. 2004). The lack of an appropriate breast cancer cell line containing significant amounts of ERβ protein can be circumvented by taking advantage of a genetic system (T47Dβ) in which a tetracycline-regulated construct drives the expression of a full-length human ERβ sequence (Ström *et al*. 2004). Here, these stably transfected T47Dβ derivatives have been used to examine the contribution of ERβ to the transcriptional reprogramming triggered by phytoestrogens.

T47Dβ cells were exposed to coumestrol (at a concentration of 1 μM) because this particular phytochemical displays the highest affinity for ERβ among

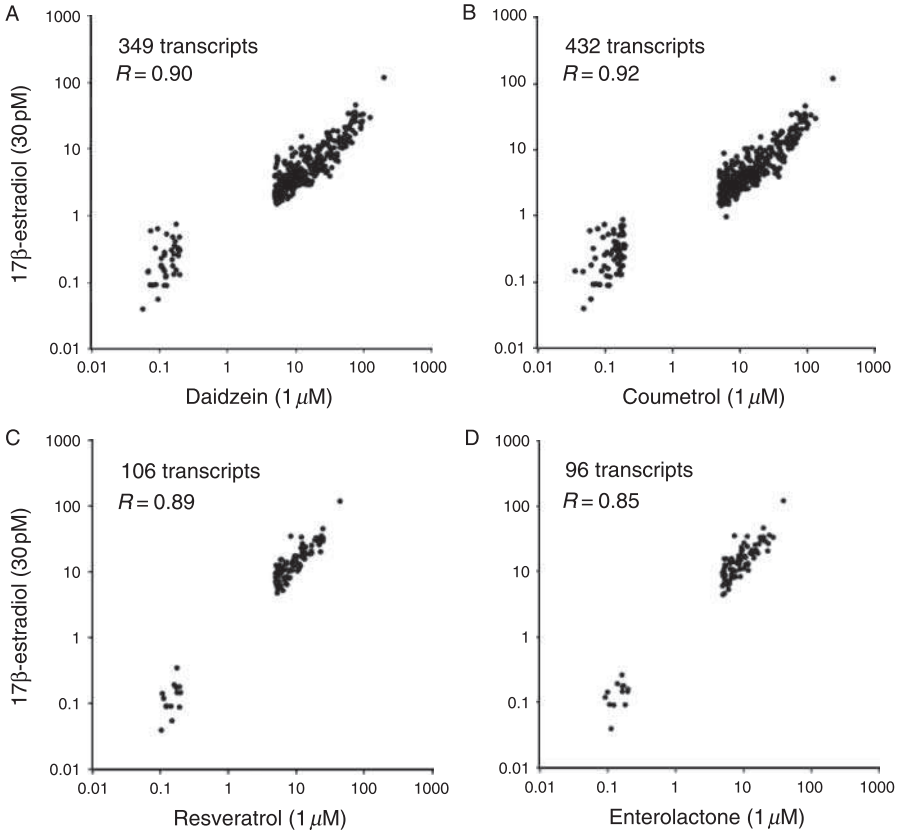

Figure 4 (A to D) Comparison between 17β-estradiol and single phytoestrogens daidzein (A), coumestrol (B), resveratrol (C), and enterolactone (D). MCF7 cells were incubated with the indicated phytochemicals at the concentration of 1 μM (three to four independent replicates). The resulting expression changes were plotted against the corresponding data obtained with 17β-estradiol (30 pM). A fivefold change in the phytoestrogen-treated samples was used as the cut-off to filter the data, and the number of significantly ($P<0.01$) regulated transcripts is indicated for each compound. The quantitative relationship between the different molecular fingerprints was determined by linear regression analyses, yielding correlation coefficients of $R=0.85$–0.92.

all phytoestrogens tested to date (Kuiper *et al*. 1997, 1998, Bovee *et al*. 2004). The transcriptional changes were tested in the presence of tetracycline, i.e., under conditions that suppress the expression of ERβ, as well as after tetracycline withdrawal, resulting in promoter activation and ERβ expression. In the absence of tetracycline, the level of mRNA coding for ERβ is four to five times higher than the corresponding ERα transcripts, thus leading to substantial quantities of both receptor subtypes (Ström *et al*. 2004). To eliminate possible confounding effects due to the antibiotic used for induction, the fingerprints obtained in the presence of tetracycline were analyzed against appropriate solvent controls containing the same level of antibiotic. Conversely, the fingerprints obtained after tetracycline withdrawal, promoting ERβ expression, were determined against corresponding controls without tetracycline in the medium.

Figure 5 illustrates the distinctly different transcriptional profiles induced by coumestrol in the absence or in the presence of ERβ. To simplify the representation of data, the graph of Fig. 5 shows only those transcripts that were up-regulated by a fold change >10 in at least one of the treatment groups ($P<0.01$ for all transcripts). The response obtained in T47Dβ cells containing ERα alone involves, for example, an overexpression of interleukin 20 (IL 20), chemokine ligand 12 (CXCL2), insulin-like growth factor-binding protein-4 (IGFBP4), MYB or trefoil factor 1 (TFF 1 also known as pS2). The range of regulated target genes did not change when, in the absence of tetracycline, both ERα and ERβ were expressed in the same cell line. However, the induction of many transcripts was attenuated in the presence of ERβ (Fig. 5), indicating that this additional receptor subtype is able to partially suppress some transactivation functions mediated by ERα. Conversely, in the presence of ERβ, other transcripts were regulated with larger amplitudes of induction than in the cells expressing only ERα. This second category of genes,

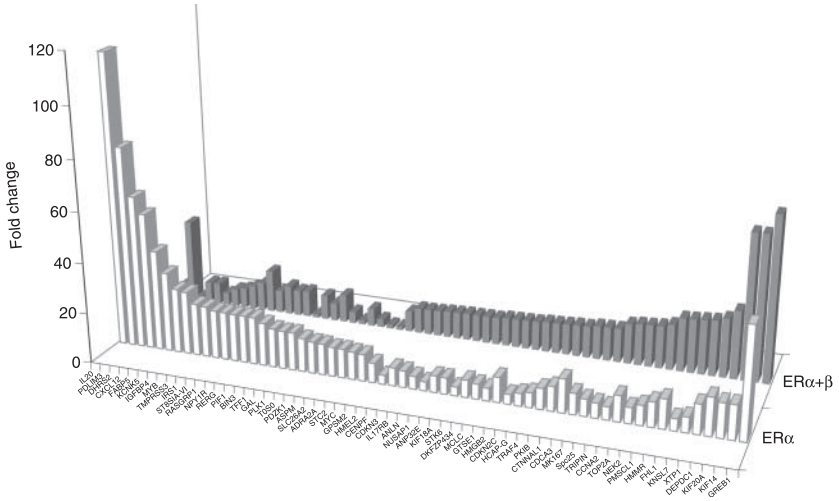

Figure 5 Impact of ERβ on gene expression profiles in breast cancer cells. T47Dβ cells were exposed to coumestrol (1 μM) either in the presence or in the absence of tetracycline. The fold changes of each transcript (mean values of three independent experiments) were calculated using, as the reference, solvent controls with or without tetracycline. ERα gene expression profile induced in cells containing only ERα (in the presence of tetracycline). ERα + β gene expression profile induced in cells containing both ERα and ERβ (in the absence of tetracycline). IL20, interleukin 20; PDLIM3, PDZ and LIM domain 3; DHRS2, dehydrogenase/reductase (SDR family) member 2; CXCL12, chemokine ligand 12; FABP5, fatty acid binding protein 5; KCNK5, potassium channel, subfamily K, member 5; IGFBP4, IGF binding protein 4; MYB, myeloblastosis oncogene; TMPRSS3, transmembrane protease serine 3; IRS1, insulin receptor substrate 1; ST8SIA-VI, α-2,8-sialyltransferase; RASGRP1, RAS guanyl releasing protein 1; NPY1R, neuropeptide Y receptor 1; RERG, RAS-like estrogen-regulated growth inhibitor; PIF1, DNA helicase homolog 1; BIN3, bridging integrator 3; TFF1, trefoil factor 1; GAL, galanin; PLK1, polo-like kinase 1; TOSO, regulator of Fas-induced apoptosis; PDZK1, PDZ domain containing 1; ASPM, abnormal spindle-like microcephaly; SLC26A2, solute carrier family 26 member 2; ADRA2A, adrenergic α-2A receptor; STC2, stanniocalcin 2; MYC, myelocytomatosis oncogene; GPSM2, G-protein signaling modulator 2; CENPF, centromere protein F; CDKN3, cyclin-dependent kinase inhibitor 3; IL17RB, interleukin 17 receptor B; ANLN, anilin; NUSAP1, nucleolar and spindle associated protein 1; ANP32E, acidic nuclear phosphoprotein 32 family E; RACBAP1, Rac GTPase-activating protein 1; STK6, serine–threonine kinase 6; KIF18A, kinesin family member 18A; MCLC, mid-1-related chloride channel 1; GTSE1, G2 and S phase-expressed 1; HMGB2, high-mobility group box 2; CDKN2C, cyclin-dependent kinase inhibitor 2C; HCAP-G, chromosome condensation protein G; TRAF4, TRAF4-associated factor 1; PKIB, protein kinase inhibitor-β; CTNNAL1, catenin α-like 1; CDCA3, cell division cycle associated 3; MKI67, antigen identified by monoclonal antibody Ki-67; Spc25, kinetochore protein Spc25; CCNA2, cyclin A2; TOP2A, topoisomerase II-α; NEK2, never in mitosis gene a-related kinase 2; PMSCL1, polymyositis–scleroderma autoantigen 1; HMMR, hyaluronan-mediated motility receptor; FHL1, four-and-a-half LIM domains 1; KNSL7, kinesin-like 2; XTP1, HBxAG transactivated protein 1; DEPDC1, DEP domain containing 1; KIF20A, kinesin family member 20A; KIF14, kinesin family member 14; GREB1, GREB1 protein.

which become more responsive in the presence of ERβ, include, for example, tripin, TOP2A (see legend to Fig. 5 for abbreviations), the cyclin-dependent kinase inhibitors CDKN3 and CDKN2C as well as multiple members of the kinesin family (KNSL7, KIF20A, KIF14).

Toward a distinctive phytoestrogen signature

Many phytoestrogens display a selective binding to ERβ whereas 17β-estradiol has approximately the same affinity for both major ER subtypes (Kuiper *et al.* 1997, 1998). Thus, we explored the hypothesis that the preferential interaction of these phytochemicals with ERβ may mediate a more distinctive transactivation function. These experiments were again carried out with coumestrol because of its superior affinity for ERβ compared with other phytoestrogens (Bovee *et al.* 2004). The expression changes were subjected to a filter for transcripts that exhibit at least a threefold induction relative to untreated controls. A statistical threshold of $P<0.05$ was applied to all differentially expressed transcripts.

In T47Dβ cells containing only ERα, in the presence of tetracycline, we observed the usual tight correlation between the expression profiles generated by 17β-estradiol and coumestrol, with 79 transcripts matching the filtering criteria (Fig. 6A). Upon linear regression analysis, the correlation coefficient between the 17β-estradiol and coumestrol data reached a value of $R=0.90$. For the same transcripts, however, this correlation coefficient was reduced to $R=0.70$ when the cells, in the absence of tetracycline, were able to express the ERβ subtype (Fig. 6B). Also, the slope of the linear regression decreased substantially from 0.7

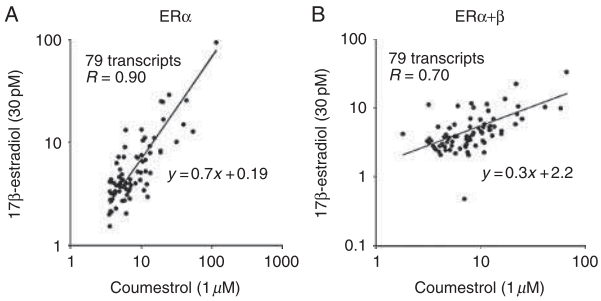

Figure 6 Diverging expression profiles in the presence of ERβ. T47Dβ cells were challenged with 17β-estradiol (30 pM) or coumestrol (1 μM), either in the presence or in the absence of tetracycline. The fold changes of each transcript (mean values of three independent experiments) have been calculated using, as the reference, solvent controls with or without tetracycline. (A) Comparison of gene expression profile in cells containing only ERα (in the presence of tetracycline). (B) Comparison of expression profiles in cells containing both ERα and ERβ (in the absence of tetracycline). These graphs illustrate that ERβ decreases both the correlation coefficient and the slope of the linear regression.

(Fig. 6A) to 0.3 (Fig. 6B), reflecting a diminished response to 17β-estradiol, relative to the effects of coumestrol, in cells expressing ERβ. Thus, the similarity of transcriptomic patterns generated by 17β-estradiol and coumestrol is less pronounced in the presence of ERβ, confirming the notion that this particular receptor subtype may mediate differential cellular responses when it is stimulated by phytoestrogens.

Discussion

Breast cancer has become the most common malignancy among American and European women (Rice & Whitehead 2006) but, in eastern countries such as Japan, the incidence of breast cancer is only about one-third that of western populations. This difference has often been attributed to a much higher dietary intake of soy phytoestrogens (Konstantakopoulos *et al.* 2006, Martinez *et al.* 2006, McCarty 2006, Messina *et al.* 2006). However, despite the large amount of research conducted in the last years, no clear consensus has emerged regarding the preventive action of phytoestrogens against cancer. There is still no conclusive evidence that the ingestion of phytoestrogens is directly related to a reduced incidence of breast cancer, or whether phytoestrogens rather represent a biomarker of generally healthy diets (Martinez *et al.* 2006, Rice & Whitehead 2006).

At low physiologic serum concentrations that are normally achieved by nutritional intake, phytoestrogens are likely to act through modulation of estrogen signaling. In fact, these lower serum concentrations appear insufficient to inhibit tyrosine kinases or other enzymes that may provide alternative targets of phytoestrogen effects (McCarty 2006). Most estrogenic responses are mediated by two members of the nuclear steroid receptor superfamily, i.e., ERα and ERβ. Both receptors constitute ligand-stimulated transcription factors that associate with co-regulatory partners to remodel chromatin and recruit the general transcription machinery to downstream genes (Katzenellenbogen & Katzenellenbogen 2000, Hall *et al.* 2001, Moggs & Orphanides 2001, Safe 2001). Although both receptors bind to the same consensus estrogen-responsive element within gene promoters, ERα and ERβ have been shown to exert partially antagonistic effects (Omoto *et al.* 2003, Ström *et al.* 2004).

Several findings converge on the idea that the proliferative stimulus mediated by activation of ERα can be opposed by the expression of ERβ. First, the knockout mice lacking ERβ are more susceptible than wild-type controls to develop markers of epithelial hyperplasia in the mammary gland (Förster *et al.* 2002). Second, the mRNA coding for ERα is up-regulated during cancer progression, whereas the ERβ transcript is reduced in part via promoter methylation (Iwao *et al.* 2000, Rutherford *et al.* 2000, Rody *et al.* 2005, Park *et al.* 2006). Third, the continued expression of ERβ in breast tumors is associated with low aggressiveness and improved survival rates compared with ERβ-negative counterparts (Hopp *et al.* 2004). Fourth, the activation of ERα promotes the growth of breast cancer cells both in culture and in animal models (Soto *et al.* 1995, Hsie *et al.* 1998, Allred *et al.* 2001, Ju *et al.* 2006) but, when ERβ is restored using an appropriate expression vector, it exerts a negative effect on cell proliferation or even induces apoptosis (Omoto *et al.* 2003, Skliris *et al.* 2003, Ström *et al.* 2004). This antiproliferative action of ERβ correlates with the down-regulation of several factors involved in DNA replication and the cell cycle machinery (Lin *et al.* 2007).

Contrary to 17β-estradiol, which does not discriminate between ERα and ERβ, phytoestrogens bind to ERβ with up to five times higher affinities compared with ERα (Kuiper *et al.* 1997, 1998). This finding appears relevant in view of the possible action of ERβ as a tumor suppressor because phytoestrogens may be able to trigger beneficial responses through their preferential interaction with the ERβ subtype. However, such a protective effect is abrogated in cells that specialize on the expression of ERα with minimal residual amounts of ERβ. This possible mechanism was supported when, in the present report, we analyzed the transcriptional fingerprints induced by soymilk extracts in human breast cancer cells. This type of soy product has been tested in the context of

this study because of its high content in isoflavones with estrogenic activity, such that the findings may be extrapolated to a wide range of other soy-based foods and supplements. Unlike previous reports (Wang *et al*. 2004, Ise *et al*. 2005), we observed that this natural mixture of soy phytoestrogens, as well as other types of phytoestrogens, induces a stereotyped expression fingerprint in breast cancer cells containing high levels of ERα but essentially no ERβ. Thus, in the absence of ERβ, all tested phytoestrogens result in essentially the same expression changes as those induced by the endogenous 17β-estradiol, and this recurrent genomic profile reflects the proliferative response mediated through ERα activation. This particular signature includes the up-regulation of multiple factors involved in cell cycle, DNA replication, chromosome segregation, and inhibition of apoptosis. However, when the expression of ERβ is reconstituted using an inducible genetic system, the same breast cancer cells react in a different manner to phytoestrogen stimulation. First, the induction of many growth-promoting transcripts involved in the cell division cycle is attenuated compared with the genuine 17β-estradiol response and second, there is a stronger induction of factors that arrest cell proliferation, such as, for example, inhibitors of cyclin-dependent kinases, thus further contributing to the inhibition of cell proliferation.

We conclude that this attenuation of ERα-induced expression fingerprints may account for the presumed chemopreventive activity of phytoestrogens since, as indicated before, many of these compounds display particularly strong affinities for ERβ. In view of our findings, we propose a biphasic activity of phytoestrogens during cancer development in estrogen-sensitive tissues. Thus, the presumed beneficial effects of phytoestrogens depend on the timing of exposure. Initially, phytoestrogens are able to slow down cell growth by activating ERβ, thereby generating an anti-proliferative expression signature. Due to the genetic instability of malignant tumor cells, however, the expression of ERβ may be abrogated by gene deletion or promoter methylation. In such late-stage cancer cells, phytoestrogens, in conjunction with other estrogenic chemicals, induce a transcriptional profile that promotes the proliferation of those clones that exhibit high amounts of ERα but little ERβ. In light of these considerations, the potentially beneficial effect of phytoestrogens should be reevaluated, particularly in relation to risk groups that are susceptible to the development of ERα-positive tumors arising from steroid hormone-dependent tissues.

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4 Selective regulation of nuclear orphan receptors 4A by adenosine receptor subtypes in human mast cells

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Contribution of Ramiro Dip

Conceived the project and recruited the necessary funds; designed and supervised all experiments; performed the transcriptomics experiments; analysed the data; wrote the manuscript. Corresponding author.

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RESEARCH ARTICLE

Selective regulation of nuclear orphan receptors 4A by adenosine receptor subtypes in human mast cells

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Abstract Nuclear orphan receptors 4A (NR4A) are early responsive genes that belong to the superfamily of hormone receptors and comprise NR4A1, NR4A2 and NR4A3. They have been associated to transcriptional activation of multiple genes involved in inflammation, apoptosis and cell cycle control. Here, we establish a link between NR4As and adenosine, a paradoxical inflammatory molecule that can contribute to persistence of inflammation or mediate inflammatory shutdown. Transcriptomics screening of the human mast cell-line HMC-1 revealed a sharp induction of transcriptionally active NR4A2 and NR4A3 by the adenosine analogue NECA. The concomitant treatment of NECA and the adenosine receptor A_{2A} (A_{2A}AR) selective antagonist SCH-58261 exaggerated this effect, suggesting that upregulation of these factors in mast cells is mediated by other AR subtypes (A_{2B} and A_3) and that A_{2A}AR activation counteracts NR4A2 and NR4A3 induction. In agreement with this, A_{2A}AR-silencing amplified NR4A induction by NECA. Interestingly, a similar A_{2A}AR modulatory effect was observed on ERK1/2 phosphorylation because A_{2A}AR blockage exacerbated NECA-mediated phosphorylation of ERK1/2. In addition, PKC or MEK1/2 inhibition prevented ERK1/2 phosphorylation and antagonized AR-mediated induction of NR4A2 and NR4A3, suggesting the involvement of these kinases in AR to NR4A signaling. Finally, we observed that selective A_{2A}AR activation with CGS-21680 blocked PMA-induced ERK1/2 phosphorylation and modulated the overexpression of functional nuclear orphan receptors 4A. Taken together, these results establish a novel PKC/ERK/nuclear orphan receptors 4A axis for adenosinergic signaling in mast cells, which can be modulated by A_{2A}AR activation, not only in the context of adenosine but of other mast cell activating stimuli as well.

Keywords Nuclear orphan receptor 4A · Mast cells · Adenosine · Adenosine receptor

Abbreviations

AR	Adenosine receptor
HMC-1	Human mast cell line 1
NBRE-LUC	Nerve-growth factor I-B responsive element-luciferase reporter gene
NR4A1	Nuclear orphan receptor 4 A1
NR4A2	Nuclear orphan receptor 4 A2
NR4A3	Nuclear orphan receptor 4 A3
PKC	Protein kinase C
RT-PCR	Reverse transcriptase polymerase chain reaction

Concise summary Non-selective AR engagement triggers robust induction NR4A orphan receptors in mast cells. Induction of these early responsive genes can be blocked by selective activation of the anti-inflammatory A_{2A}AR not only in the context of adenosine but also of PMA signaling, suggesting a general mechanism of mast cell regulation.

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Introduction

NR4A orphan receptors are transcription factors that belong to the superfamily of steroid nuclear hormone receptors that have been associated with different cellular processes, including inflammation (Murphy et al. 2001; Pei et al. 2005), steroidogenesis (Manna et al. 2002), apoptosis (Winoto and Littman 2002), development of dopaminergic

neurons (Zetterstrom et al. 1997) and glucose metabolism (Pei et al. 2006b). This subfamily of nuclear orphan receptors is constituted by 3 members: NR4A1 (also known as Nur77 or nerve growth factor inducible B, NGFI-B), NR4A2 (also known as Nurr1) and NR4A3 (also known as NOR1). In the context of inflammation, these factors represent NF-κB and LPS-inducible genes in macrophages (Pei et al. 2005) and NR4A1 expression leads to induction of inflammatory genes, potentiating NF-κB pro-inflammatory signaling by IKKi kinase upregulation (Pei et al. 2006a). In addition, NR4A2 has been recognized as an important inducible factor in inflamed synovium and as a target of anti-inflammatory effects of methotrexate (Ralph et al. 2005).

Adenosine is a purine nucleoside normally present in the nanomolar range but its concentration in the extracellular space rises with increased oxygen consumption during hypoxia, tissue injury and inflammation. The rapid accumulation of adenosine is followed by biological responses through activation of 4 types of G-coupled adenosine receptors (ARs): A_1, A_{2A}, A_{2B} and A_3. Each of the receptor subtypes has a different pharmacological profile, tissue distribution and effector coupling profile. During chronic inflammatory processes the sustained formation of adenosine has been associated with deleterious effects. Elevated adenosine concentrations can be found, for example, in bronchoalveolar lavage and exhaled breath condesate of human patients with asthma (Driver et al. 1993; Huszar et al. 2002) where it perpetuates inflammation and contributes to airway hyperresponsiveness. However, activation of the A_{2A}AR has been associated to the suppression of inflammation and tissue remodelling (Fozard 2003; Ohta and Sitkovsky 2001) and inhibition of histamine and tryptase release from human mast cells (Suzuki et al. 1998). However, the transcriptional effectors downstream of AR activation responsible for adenosine's pro or anti-inflammatory effects remain to be characterized.

In this study, we employed the human mast cell HMC-1 to characterize subtype specific effectors of AR activation and we established a link between adenosine receptor activation and nuclear orphan receptors. Furthermore, we determined that activation of the A_{2A}AR counterbalances the induction of these transcription factors and that this effect is not limited to adenosinergic signaling but that it is also conserved for other mast cell activating stimuli. These observations describe a novel regulatory mechanism by A_{2A}AR, with implications in the progression of inflammation and related pathologies.

Materials and methods

Reagents and cell culture

All chemicals were obtained from Sigma-Aldrich (Switzerland) unless otherwise indicated. The human mast cell line-1 (HMC-1) was a kind gift from Dr. J. H. Butterfield, Mayo Clinic, Rochester, MN, USA and was grown in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen) supplemented with 10% iron supplemented fetal bovine serum (Invitrogen), 1.2 mM alphathiglycerol (Sigma) and 100'000 U/l Penicillin and 100 mg/l Streptomycin (complete IMDM) at 37°C in 5% CO_2.

Cell treatments

HMC-1 cells were seeded at 8×10^5 cells/ml and allowed to settle overnight. They were then treated at the indicated concentrations with the following chemicals: the adenosine analogue NECA (5′-N-Ethylcarboxamido-adenosine), the A_{2A}AR agonist CGS-21680 (9-Chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine), the A_{2A}AR antagonist SCH-58261 (7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine) and PMA (phorbol 12-myristate 13-acetate). The length of treatment varied between 30 min and 72 h. Cells were pretreated with 1 U/ml adenosine deaminase (Roche) to remove any pre-existing endogenous adenosine for 20 min.

RNA extraction and cDNA synthesis

Cells were collected by centrifugation at the corresponding time points and total RNA was recovered using the Qiashredder and Rneasy mini kit (Qiagen). Concentration and quality of total RNA were measured with the Ultraspec 2100 pro spectrophotometer (Amersham Biosciences). Samples with a UV absorbance 260/280 ratio of 1.8–2.1 were considered to be suitable for cDNA synthesis. RNA samples were stored at −20°C until use. Complementary DNA (cDNA) was synthesized using the one-cycle cDNA synthesis kit followed by a sample cleanup to optimize volumes and concentration of the cDNA (GeneChip sample cleanup module, Affymetrix).

Genome wide gene expression analysis

Global gene expression analysis was done in 4 independent experiments, consisting of the treatment with 10 μM NECA and untreated cells as a baseline control. After 3 h treatment of HMC-1 cells total RNA was extracted as described in the previous section. The quality of RNA was determined on the Agilent Lab-on-a-chip Bioanalyzer 2000 (Palo Alto, USA). Samples with a total area under 28S and 18S bands of less than 65% of total RNA, as well as a 28S/18S ratio of less than 1.5, were considered to be degraded and therefore excluded from microarray analysis. cRNA was synthesized from cDNA with the IVT labeling kit (Affymetrix). cRNA quality was assessed with the Agilent Lab-on-a-chip

Bioanalyzer 2000. The biotin-labeled cRNA was fragmented and hybridized on Human Genome U122 plus 2.0 microarrays (Affymetrix), which cover sequences of 47'000 human transcripts, following the manufacturer's instructions. After hybridization periods of 16 h the microarrays were automatically washed and stained on the Affymetrix Fluidics Station 450. Staining of the hybridized probes was performed with fluorescent streptavidin-phycoerythrin conjugates (1 mg/ml; Molecular Probes). The subsequent scanning of DNA microarrays was carried out on an Affymetrix scanner 3000 7 G. The generated data was then normalized and subsequently filtered using a significance value of $P< 0.05$ using the GeneSpring 7.3.1 sofware (Agilent, Palo Alto, CA, USA). We employed pair-wise analysis based on B - Fabric infrastructure tool (Functional Genomics Center Zurich, University of Zurich-Irchel, Switzerland) and the GeneGo Metacore (www.genego.com) integrated software for data mining and functional analysis of experimental data. Hierarchical clustering was performed with gplots library from CRAN.

Real time polymerase chain reaction (RT-PCR)

Specific primers for the selected transcripts as well as TaqMan probes and TaqMan master mix were obtained from Applied Biosystems. 40 ng of cDNA were mixed with 1 μl of forward and reverse primers and 10 μl of master mix supplemented with 25 nM of the corresponding TaqMan probe in a final volume of 20 μl. The reactions were performed in a 7500 Fast Real-time PCR-System ABI 7500 (Applied Biosystems) in 40 cycles (95°C for 3 s, 60°C for 30 s) after an initial 20 s incubation at 95°C, and was analyzed with the 7500 Fast System SDS Software System (Applied Biosystems). The fold change in expression of each gene was calculated with the 2-Delta Delta C(T) method. Each of these values had been normalized to the endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western blot

Thirty microgram of total protein in 1× Laemmli buffer were separated on a 10% polyacrylamide gel by standard SDS-PAGE technique from each sample, followed by transfer onto Immune-Blot polyvinylidene difluoride (PVDF) membranes (0.2 μm pore size, Bio-Rad) and blocking 16 h at 4°C with blocking solution (5% non-fat dry milk, 3% BSA in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.4). NR4A2 and total ERK protein levels were assessed employing a monoclonal antibody (Alpha Diagnostic and R&D, respectively) and phosphorylated ERK1/2 by a specific T185/Y187 phopsho-antibody (R&D, USA). GAPDH and β-actin antibodies (Ambion, USA) were employed as internal loading controls. Enhanced chemiluminescence was performed with SuperSignal West Femto Maximum (Thermo Fisher Scientific, Switzerland) and images were acquired on a LAS-3000 image reader (Fujifilm Life Science, Japan). NR4A2 induction was quantified by calculating the ratio of intensity signals to GAPDH (Quantity 1 software, BioRad) and compared to untreated controls (value=1).

siRNA silencing of $A_{2A}AR$

0.4×10^6 HMC-1 cells in 1 ml were seeded in 24 well-plates. Cells were transcfected with 20nM $A_{2A}AR$ siRNA (Santa Cruz, Cat. sc-39850) using 4 μl lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions in Opti-MEM serum and antibiotics-free medium. After 5 h of incubation, the medium was replaced with complete medium. As a scramble negative control, control (FITC Conjugate)-A siRNA (Santa Cruz, sc-36869) was employed. Silencing of ARs was tested by RT-PCR with specific TaqMan probes (Applied Biosystems) as indicated above, and was maximal at 36 h. Therefore, this time point was selected for further analysis of AR signaling.

Transient transfection and luciferase reporter gene assay

1.5×10^6 HMC-1 cells in 1 ml were co-transfected with the tk-NBREx3-luc plasmid, which was a kind gift Dr. R Evans (Howard Hughes Medical Institute, San Diego, CA) and the pRL-TK vector (Promega, USA) at a ratio 30:1 in serum- and antibiotics-free Iscove's medium containing 8 μl of Lipofectamine 2000 reagent (Invitrogen). After 5 h of incubation, the medium was replaced with complete medium and were allowed to recover at 37°C for 24 h and were subsequently stimulated as indicated. For luciferase activity assay, cell lysates were prepared and assayed using the Luciferase Assay System (Promega, USA), according to the manufacturer's instructions. All luminescent measurements were performed automatically in a 96 well-plate in a Luminescence Spectrometer (MLX, Dynex).

Data analysis

Statistical analysis was performed using the PASW Statistics software (SPSS Inc., USA). Data were analyzed by nonparametric tests (Mann-Whitney U-test) and differences between groups were considered significant when p-values were less than 0.05.

Results

Transcriptional profiling of human mast cells reveals upregulation of NR4A family memebers by adenosine receptor activation

The human mast cell line HMC-1 exhibits a phenotype, which in several aspects is similar to tissue resident mast cells (Nilsson et al. 1994). However, these cells do not degranulate and therefore represent a suitable system for studying degranulation-independent de novo synthesis of inflammatory mediators.

Previous studies have successfully used this cell line to analyze adenosine signaling on human mast cells (Ryzhov et al. 2006; Ryzhov et al. 2004), which prompted us to employ a systematic genome-wide approach for the identification of novel effector molecules involved in AR signaling. For this, we stimulated HMC-1 cells to the adenosine analogue NECA and 3 h after exposure the total fraction of RNA transcripts was extracted, processed and hybridized to Affymetrix microarrays (4 replicates). To identify differentially expressed genes, the data sets of each replicate were analysed in a pair-wise fashion with the untreated controls as described in the methods section. This analysis yielded 19 NECA-induced transcripts with fold changes of three or higher (Table 1). The resulting heat map reflects the degree of similarity between the individual replicates (vertical dendogram, Fig. 1a). Gene ontology analysis of these transcripts revealed that most of these transcripts encode protein products involved in transcription regulation (NR4A2, NR4A3, CREM , EGR1, 2 and 3, FOSB, ELL2, ZEB1), signal transduction (HOMER1, ITK, IL2RB), hormone activity (STC1, CGA), DNA metabolism and repair (JMY, TP53INP2 ITK), cell cycle regulation (RGC32), apoptosis (BCL2L11) and tissue remodeling (PLAUR). Remarkably, two nuclear orphan receptors (NR4A2 and NR4A3), which had not been associated to adenosine signaling before, were among the highest upregulated genes. Interestingly, the third member of orphan receptor family, NR4A1, was induced only marginally (2.05 fold). These results were confirmed by real time RT-PCR (Fig. 1b) and suggest that NR4A2 and NR4A3 could mediate proinflammatory signaling by adenosine in mast cells.

A_{2A}AR inactivation amplifies NECA-mediated induction of NR4A2 and NR4A3

Human mast cells express A_{2A}, A_{2B}, and A_3ARs (Feoktistov and Biaggioni 1998; Feoktistov et al. 2003). Therefore, the observed response to NECA reflects the composite effect of activation of all 3 receptor subtypes. Because AR subtypes can promote (A_{2B} and A_3ARs) or downregulate (A_{2A}AR) inflammation, we wanted to determine the influence of A_{2A}AR in NR4A induction. To address this issue cells were stimulated either with NECA, with the selective A_{2A}AR agonist CGS-21680, or with a combination of NECA plus the A_{2A}AR antagonist SCH-58261, and NR4A induction

Table 1 Top regulated transcripts by non-selective AR stimulation. Cells were treated with 10 μM NECA and fold changes (FC) calculated against untreated control as described (fold change cut-off=3, P<0.005)

Gene Name	Description	NECA FC
NR4A2	Nuclear receptor subfamily 4, group A, member 2	33.52
STC1	Stanniocalcin 1	25.37
NR4A3	Nuclear receptor subfamily 4, group A, member 3	24.13
CREM	CAMP responsive element modulator	13.57
EGR3	Early growth response 3	10.16
RGC32	Response gene to complement 32	9.54
HOMER1	Homer homolog 1 (Drosophila)	7.95
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	6.91
CGA	Glycoprotein hormones, alpha polypeptide	6.14
ELL2	Elongation factor, RNA polymerase II, 2	6.14
JMY	Junction-mediating and regulatory protein	5.96
ZEB1	Transcription factor 8	5.42
EGR1	Early growth response 1	5.07
BCL2L11	BCL2-like 11 (apoptosis facilitator)	4.99
ITK	IL2-inducible T-cell kinase	4.76
PLAUR	Plasminogen activator, urokinase receptor	4.58
TP53INP2	p53 induced nuclear protein 2	3.88
IL2RB	Interleukin 2 receptor, beta	3.78
EGR2	Early growth response 2 (Krox-20 homolog, Drosophila)	3.72

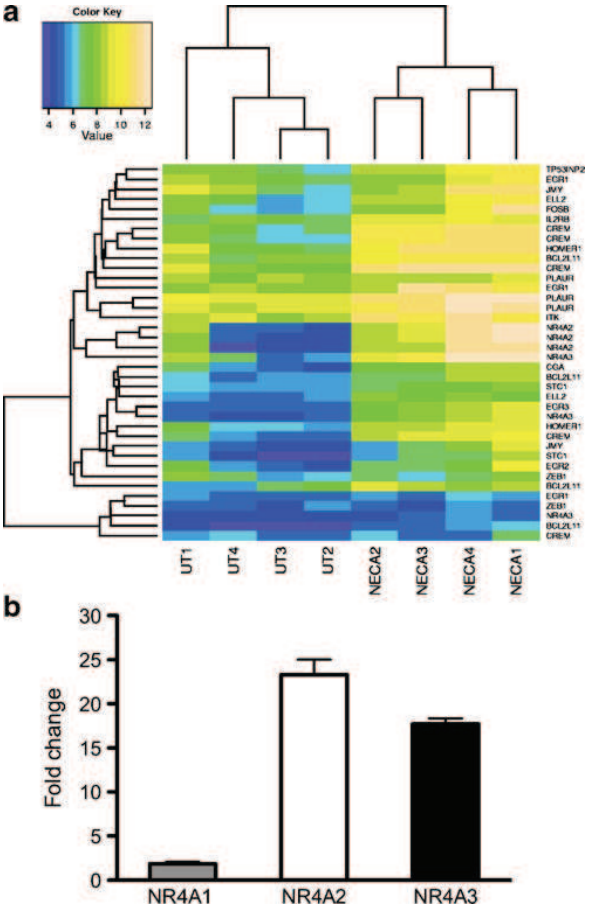

Fig. 1 **a** Hierarchical clustering analysis of transcriptional changes of HMC-1 cells induced by NECA. Each column represents a single experiment (4 replicates for each condition, untreated and treated with 10 μMNECA, 3 h) and contains all genes that were significantly upregulated in at least one of replicates and a minimum induction of 3 fold. The resulting vertical dendrogram indicates the degree of similarity between different transcriptomes. Expression levels are expressed in a log 2 scale and transcripts with values of 5 or lower (blue and light blue) are marginally expressed or absent. Repeated gene names indicate different probe sets for a single gene (different expression signals for alternative probe sets could indicate alternative splicing or poor probe performance). **b** Confirmation of NR4A induction by RT-PCR. Fold changes are calculated in relation to untreated controls. Values represent the mean of three experiments ± SEM

was assessed by RT-PCR. Figure 2a and b show time-dependent induction of NR4A2 and NR4A3. While NECA-mediated induction of NR4A2 and NR4A3 reached maximal induction of 240-fold and 98-fold respectively 1 h after treatment, selective activation of A_{2A}AR with 1 μM CGS-21680 did not induce them. In contrast, blockage of A_{2A}AR with the combined treatment of NECA and SCH-28261, results in further upregulation of these nuclear receptors, with fold changes up to 429 and 376 over the untreated controls respectively. These observations point out that the induction of these two transcription factors rely on the activation of *non-A_{2A} ARs*. Interestingly, NECA plus SCH-58261 did not upregulate NR4A1 further when compared to NECA (4.2-fold, Fig. 2c), indicating that this interplay between non-selective and selective A_{2A}AR activation is not conserved to the third member of this family of receptors. Also, induction of the cAMP-response element modulator (CREM, a top-regulated gene from the transcriptomics screening, see Table 1) reaches 55-fold when treated with NECA, while CGS-21680 or the combination of NECA plus SCH-58261 induce CREM only marginally, indicating that simultaneous activation of all ARs is required to achieve maximal CREM induction (Fig. 2d).

To confirm the role of A_{2A}AR dependent changes in NR4A2 and NR4A3 expression, HMC-1 cells were transfected with A_{2A}AR-specific siRNA, which led to 63% silencing 36 h after transfection (Fig. 3a). Treatment of A_{2A}AR silenced cells with NECA resulted in higher NR4A2 expression levels as compared to mock silenced cells, mimicking the effect of pharmacological blockage of A_{2A}AR with the combination of NECA plus SCH-58261 (Fig. 3b). Altogether, these results indicate that activation of A_{2A}AR counterbalances NR4A2 and NR4A3 induction by A_{2B}AR and A_3AR.

Adenosine receptor activation increases NR4A2 protein abundance

Next, we wanted to determine whether AR-mediated increment of NR4A2 mRNA levels translate into changes in protein abundance. Western blot analysis of NR4A2 protein revealed that NECA treatment results in a remarkable induction of this factors reaching maximal levels at 12 h, with values returning to background levels 24 h after exposure (Fig. 4). As expected, selective A_{2A}AR activation with CGS-21680 did not upregulate NR4A2 (not shown). However, induction of NR4A2 protein by the concomitant treatment of NECA plus SCH-58261 was higher than with NECA but also returned to basal levels within 24 h. These results confirm that transcriptional upregulation of NR4A2 by AR activation results in increased protein levels.

AR-induced NR4As are transcriptionally active

In view of the strong induction of NR4A2 and NR4A3, we performed a functional assay based on an exogenous reporter gene to determine how the activity of these factors is affected by AR activation. NR4A receptors bind to the octanucleotide 5′-A/TAAAGGTCA (NGFI-B response element, NBRE) and therefore we employed an NBRE-luciferase reporter plasmid (tk-NBREx3-luc) and quantified luciferase induction upon AR activation. Figure 5 shows

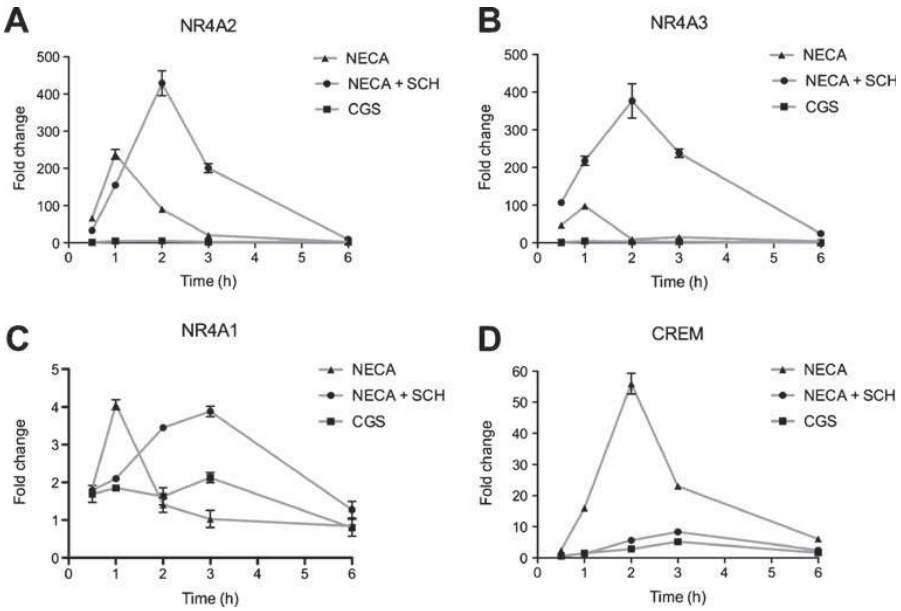

Fig. 2 Time dependent induction of NR4A2 (**a**), NR4A3 (**b**), NR4A1 (**c**) and CREM (**d**). Cells were treated with 10 μM NECA, 1 μM CGS-21680 (CGS) or 10 μM NECA plus 1 μM SCH-58261 (SCH) and RNA was collected at the indicated time points and analyzed by RT-PCR. Fold changes are calculated against untreated controls. Values represent the mean of three independent experiments ±SEM

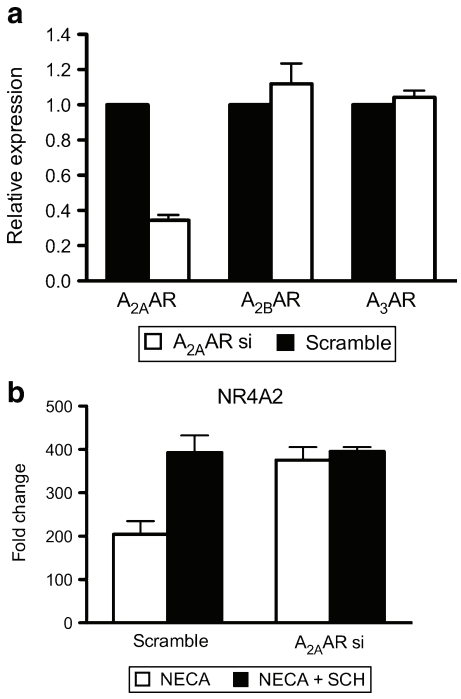

Fig. 3 Effect of $A_{2A}AR$ silencing on NR4A2 induction. **a** Transfection of HMC-1 cells with $A_{2A}AR$-siRNA ($A_{2A}AR$ si) specifically downregules this receptor subtypeation of $A_{2A}AR$. Scramble siRNA was included as a control **b** $A_{2A}AR$ silenced cells were treated with for 2 h with 10 μM of NECA or 10 μM of NECA plus 1 μM SCH-58261 (SCH). Values represent the mean of three independent experiments ± SEM

time-dependent induction of luciferase activity upon treatment with NECA, CGS-21680 or NECA in combination with SCH-58261. Both NECA and NECA plus SCH-58261 induced a robust response, which peaked between 12 h and 24 h with nearly a 30- and 40-fold reporter induction as compared to untreated controls respectively. NBRE reporter activity decreased sharply by 48 h and returned to background values 72 h after treatment. On the contrary, CGS-21680, did not induce luciferase expression significantly. Interestingly, reporter induction by NECA plus SCH-58261 was not statistically significantly higher than by NECA, despite the remarkable difference in induction of these transcription factors. This discrepancy can be explained by the intrinsic limitations of the artificial reporter gene system employed. The luciferase construct includes a minimal promoter region in the 5′-regulatory

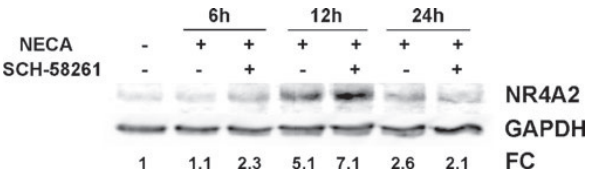

Fig. 4 Western blot analysis of NR4A2. HMC-1 cells were treated with 10 μM NECA, or the combination of 10 μM NECA and 1 μM SCH-58261 (SCH) for the indicated time points. GAPDH is included as an internal control. Fold change of NR4A2 protein (FC) are calculated from the ratio to GAPDH and compared to baseline expression in untreated cells (lane 1)

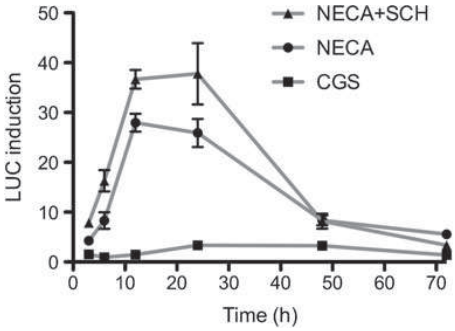

Fig. 5 Time-dependent induction of NBRE by AR engagement. The results are shown as ratio against the respective untreated control. For each measurement the ratio between the reporter gene (firefly) between and the internal Renilla standard was calculated (three independent determinations). Cells were treated with 10 μM NECA, 1 μM CGS-21680 (CGS) or 10 μM NECA plus 1 μM SCH-58261 (SCH)

region of the NBRE repeats. Therefore, this assay may not allow the full response (gene expression) that could be expected in the context of endogenous chromatin to take place, thereby limiting the amplitude of the responses measured and of the differences between treatments. Nevertheless, these results confirm that AR activation induces transcriptionally active NR4As that could have profound biological implications.

AR-mediated NR4A2 and NR4A3 upregulation involves PKC and MEK kinases, and correlates with A_{2A}AR-, PKC- and MEK-sensitive ERK1/2 phosphorylation

NR4As can be induced by a variety of stimuli, which are signaled through diverse intracellular regulators in a cell type and stimulus-dependent fashion (Martinez-Gonzalez and Badimon 2005). Therefore, we wanted to determine the intracellular signaling pathways participating in NR4A2 and NR4A3 induction by ARs. To address this issue we interrogated the role of the mitogen-activated kinase p38, protein kinase A (PKA), phosphoinositide 3 kinase (PI3K), protein kinase C (PKC) and MEK kinase with selective inhibitors, by assessing the AR-mediated induction of NR4A2 and NR4A3 (NECA plus SCH-58261). While 30 min pretreatment with SB203580 (p38 inhibitor), H89 (PKA inhibitor) and wortmannin (PI3K inhibitor) did not affect NR4A induction, blockage of either PKC or MEK with GF109203X or PD98059 respectively partially reverted AR-mediated induction of these factors (Fig. 6a). Based on previous reports that showed that ERK phosphorylation is involved in AR inflammatory signaling in HMC-1 cells (Feoktistov et al. 1999), and because ERK1/2 is a downstream target of PKC, we wanted to determine whether activation of A_{2A}AR correlates with ERK phos-

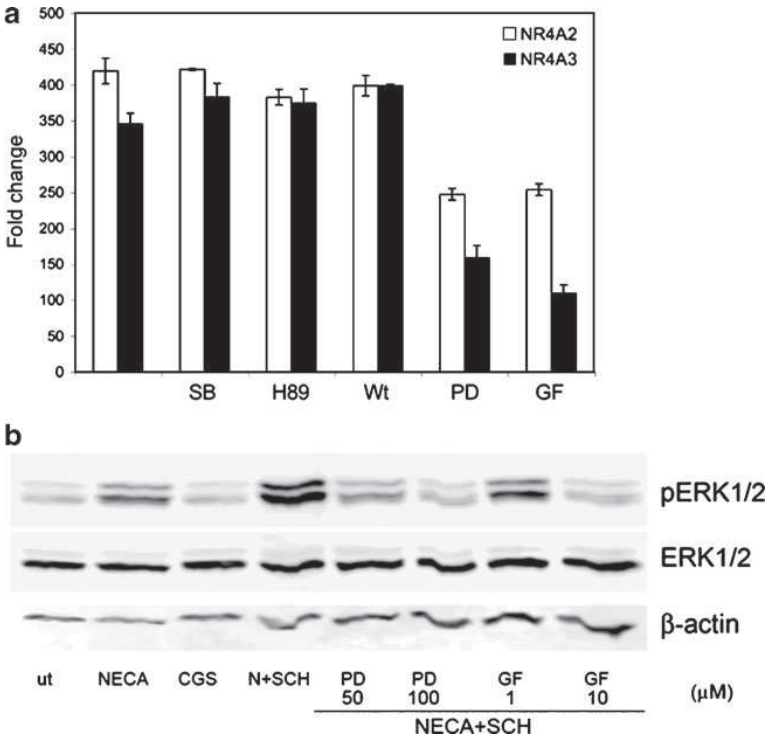

Fig. 6 Effect of kinase inhibitors on AR-mediated NR4A induction and ERK1/2 activation **a** Pretreatment with MEK and PKC inhibitors partially reversed NR4A2 and NR4A3 induction by 10 μM NECA and 1 μM SCH-58261. 0.5 μM SB203580 (SB), 1 μMH89, 0.1 μM wortmannin (Wt), 100 μM PD98059 (PD) or 10 μM GF109203X (GF) were employed for preincubation. **b** A_{2A}AR-blockage excacerbates NECA-induced phosphorylation of ERK1/2 (N+SCH). This effect is blocked by preincubation with PD98059 (PD) and GF109203X (GF) in a concentration dependent way

phorylation. NECA readily induced phosphorylation of ERK1/2 while CGS-21680 did not (Fig. 6b, lanes 2 and 3). Interestingly, NECA plus SCH-58261 resulted in exaggerated ERK1/2 phosphorylation (lane 4), demonstrating an inverse relationship between A_{2A}AR activation status and ERK1/2 phosphorylation. Furthermore, this pattern of ERK phosphorylation could be reverted by preincubation with GF109203X or with PD98059 in a concentration dependent manner (lanes 5–8). Taken together, these results indicate that PKC and MEK kinases are required for AR-dependent ERK1/2 phosphorylation and NR4A2 and NR4A3 upregulation, and that activation of A_{2A}AR opposes ERK1/2 activation by other ARs.

A_{2A}AR activation modulates PMA-induced ERK phosphorylation, NR4A2 and NR4A3 induction and NBRE transcriptional activity

Next, we wanted to assess whether A_{2A}AR activation is able to influence NR4A induction by stimuli other than adenosine. Therefore, we selected PMA, a diester of phorbol that acts as a PKC activator, which is a central regulatory molecule with a role in cytokine production, arachidonic acid release and mast cell degranulation (Chang et al. 1997; Cho et al. 2004). Stimulation of HMC-1 cells with PMA resulted in robust ERK1/2 phosphorylation that could be reverted with increasing concentrations CGS-21680 (Fig. 7a). In addition, 1 μM CGS-21680 significantly decreased PMA-mediated NR4A2 and NR4A3 induction (by 35 and 53% respectively, Fig. 7b) and, moreover, levels of PMA-induced NR4A2 protein were also reduced (Fig. 7c). In light of these findings, we tested whether A_{2A}AR activation could affect the transcriptional activity of PMA-induced NR4As, by means of the NBRE-LUC reporter assay. Remarkably, preincubation with 1 μM CGS-21680 caused a significant reduction in PMA-induced NBRE-LUC activity (35%, Fig. 7d).

Altogether, these results show that the inhibitory effect of A_{2A}AR on nuclear orphan receptor 4A stimulation is conserved beyond adenosinergic inflammatory signaling and that activation of this receptor can regulate intracellular signaling by other inflammatory stimuli.

Discussion

Mast cells have been traditionally associated to immediate-type hypersensitivity reactions through the release of preformed inflammatory mediators. However, in recent years it has become clear that these cells can regulate immune responses through de novo synthesis of cytokines, chemokines, and eicosanoids without degranulation (differential release inflammatory mediators), supporting a role of

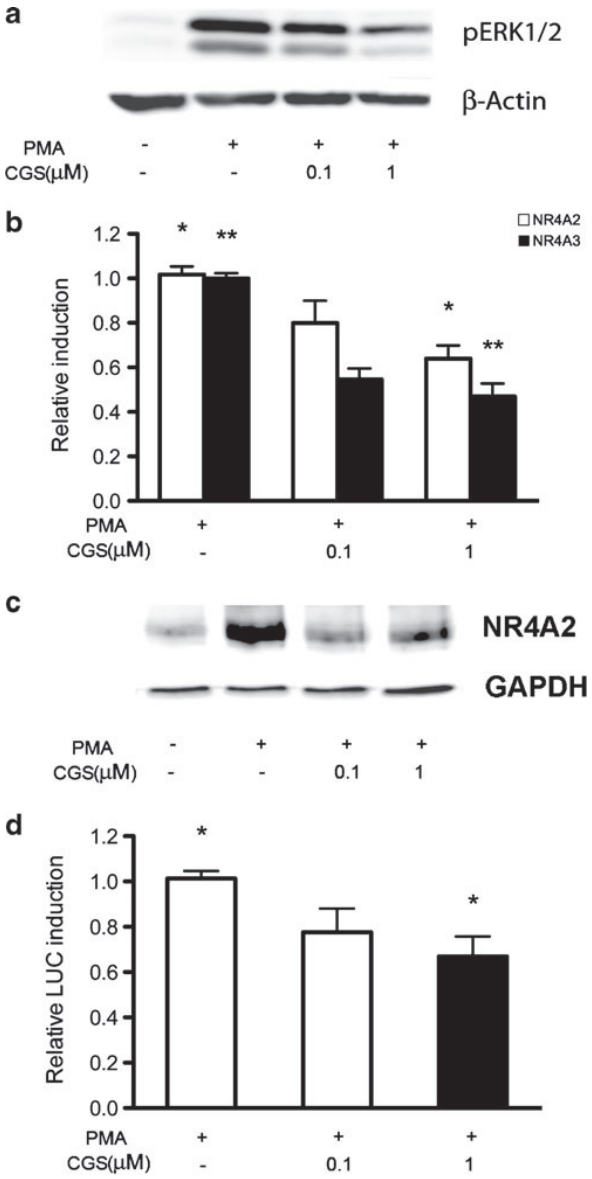

Fig. 7 Effect of A_{2A}AR activation on PMA-mediated NR4A activity. **a** Increasing concentrations of CGS-21680 (CGS) antagonize PMA-induced ERK1/2 phosphorylation. β-Actin is included as an internal control, **b** Reduction of PMA-mediated NR4A2 and NR4A3 transcriptional induction by increasing concentrations of CGS-21680 (CGS). Values are relative to induction by PMA and indicate the mean of three independent experiments ± SEM. *, **: $p<0.05$ for reduction in NR4A2 and NR4A3 induction respectively (Mann-Whitney U test), **c** Western blot analysis of NR4A2 induced by PMA alone or with the indicated concentrations of CGS-21680, **d** CGS-21680 (CGS) reduces the transcriptional activity of PMA-mediated NBRE-LUC reporter activity. For each measurement the ratio between the reporter gene (firefly) and the internal Renilla standard was calculated. Values are relative to LUC expression by PMA, and represent the mean of three independent experiments ± SEM. PMA concentration in all experiments was 50 nM. *$p<0.05$ for NBRE-LUC activity reduction (Mann-Whitney U test)

mast cells in more persistent (chronic) inflammatory and immunological responses such as chronic bronchitis and asthma-related pulmonary inflammation (Brightling et al. 2003; Church and Levi-Schaffer 1997), renal injury (Mack and Rosenkranz 2009), and tumorigenesis (Blatner et al. 2010; Groot Kormelink et al. 2009).

The engagement of activating and inhibitory cell-surface receptors, as well as the intensity and duration of these signals, will determine the activation state of mast cells; in response to these stimuli, gene expression patterns of inflammatory mediators are altered according to the sum of positive and negative signaling events, thereby affecting the course of inflammation. Based on adenosine's ability to both positively and negatively regulate mast cell activation through the engagement of alternate AR subtypes, it has been argued that selective activation of the A_{2A}AR (anti-inflammatory) or blockage of A_{2B}AR and A_3AR (proinflammatory) may represent a pharmacological tool for modulating their adenosinergic inflammatory signaling. However, transcriptional effectors downstream of ARs responsible for adenosine's pro- or anti-inflammatory effects remained to be fully characterized.

In this study we present a novel link between AR activation and NR4A orphan receptors in human mast cells, and we show that selective activation of the A_{2A}AR can negatively regulate the induction of these factors. Our initial genome-wide screening revealed strong upregulation of NR4A2 and NR4A3. Recent evidence shows that NR4A2 induction represents a common point of convergence of distinct cytokine signaling pathways, suggesting an important common role for this family of transcription factors as mediating inflammatory signaling (McEvoy et al. 2002). Therefore, we reasoned that the anti-inflammatory A_{2A}AR could influence the expression of these pro-inflammatory factors. In fact, we observed that the concomitant treatment with the A_{2A}AR antagonist SCH-58261 significantly amplified NECA's effect on NR4A2 and NR4A3 induction. In view of the pleiotropic physiological roles of NR4As, adenosine's effect on this group of transcription factors could have broad biological implications.

In contrast to other members of the nuclear hormone receptor superfamily, the crystal structure and NMR data indicate that the ligand-binding pocket of NR4A receptors is covered by hydrophobic residues (Wang et al. 2003). In fact, these receptors have been shown to function as ligand-independent transcription factors that are constitutively active and whose activity is controlled at the level of protein expression and post-translational modifications (Codina et al. 2004; Fahrner et al. 1990). For this reason, adenosine's effects on the abundance of these transcription factors could have immediate biological implications. On one hand, the activation of pro-inflammatory ARs (i.e. A_2B and A_3ARs) in inflammatory cells could act as an amplification signal resulting in even higher levels of NR4As and in the expression of a larger set of NBRE-responsive genes. On the other, A_{2A}AR activation could mediate inflammation resolution indirectly by limiting the expression of NR4A2 and NR4A3-dependent inflammatory genes.

NR4As also influence the function of other inflammation-associated transcription factors. For example, NR4A1 and NR4A2 form heterodimers with retinoic acid receptor and can influence retinoid signaling (Wallen-Mackenzie et al. 2003). Therefore, AR activation could affect the number of NR4A-containing complexes: AR-mediated accumulation of NR4A2 and NR4A3 would translate in a higher proportion of transcriptional complexes containing these orphan receptors. Conversely, A_{2A}AR activation would limit the availability of NR4A2 and NR4A3 for heterodimerization with other TFs. In addition, NR4A receptors can also crosstalk with other TFs and influence their activity without necessarily interacting with them. A recent study has established that NR4A receptors and the estrogen-related receptors NR3B mutually repress each others transcriptional activity (Lammi et al. 2007). Similarly, NR4A1 has been shown to negatively cross-talk with NF-κB (Harant and Lindley 2004). As a consequence, by virtue of the induction of reamarkably high levels of functional NR4A2 and NR4A3, adenosine is likely profoundly affect the expression of large sets of genes both directly (NBRE-responsive transcripts) and indirectly, by affecting the nature of transcriptional complexes and/or crosstalking with other TFs.

Activation of mast cells (for example by the high affinity IgE receptor) requires the activation of receptor-proximal tyrosine kinases, mobilization of internal Ca^{2+} and the formation of signaling complexes coordinated by adaptor proteins (Rivera and Gilfillan 2006). PI3K is a central player in mast cell activation that signals to regulatory molecules such as PKC and phospholipases C and D (PLC and PLD) among others, which ultimately regulate mast cell degranulation, arachidonic acid metabolite production and cytokine gene transcription. AR activation has traditionally been linked to stimulation or inhibition of the adenylyl cyclase; A_{2A}AR and A_{2B}AR activation lead to increased cAMP levels that in turn activate the canonical PKA pathway and the exchange protein directly activated by cAMP (Epac) (Palmer and Trevethick 2008), while A_1AR and A_3AR activation leads to cAMP decrease (Zhou et al. 1992). In addition, AR signaling in mast cells has also been linked to PLC and calcium mobilization (A_{2B}AR and A_3AR), PI3K (A_3AR), as well as PKC and MAP kinases (A_1, A_{2A} and A_3AR) (Jacobson and Gao 2006; Spicuzza et al. 2006).

Several intracellular regulators have been linked to NR4A induction (Martinez-Gonzalez and Badimon 2005). In this study we established that AR-mediated NR4A2 and NR4A3 upregulation in HMC-1 did not involve PKA nor

PI3K nor p38. Instead, PKC and MEK kinase inhibition could partially revert the induction of these factors and, moreover, the activity of these kinases correlated with ERK1/2 phosphorylation. Interestingly, some studies have also shown the involvement of ERKs in adenosine signaling (Feoktistov et al. 1999) but downstream targets of ERK upon AR activation had remained elusive. The results presented here suggest that activation of ERK1/2 kinases downstream of PKC mediates NR4As induction by AR. Remarkably, A_{2A}AR can counterbalance NECA-induced ERK1/2 phosphorylation, correlating with its modulatory effect on NR4A2 and NR4A3 induction. However, blockage of PKC and a complete inhibition of ERK phophorylation resulted only in about a 50% decrease NR4A2 and NR4A3 induction, suggesting the involvement of at least one additional intracellular signaling pathway in AR-dependent nuclear orphan receptor upregulation in mast cells. The contribution of other intracellular signaling pathways remains to be investigated.

Finally, we examined whether the antagonistic effect of A_{2A}AR on ERK1/2 phosphorylation and NR4A2 and NR4A3 induction is preserved to other mast cell activating stimuli, by the concomitant activation of A_{2A}AR and mast cell stimulation with PMA. Strikingly, A_{2A}AR engagement resulted not only in a modulation of PMA-mediated ERK1/2 phosphorylation, but also limited NR4A2 and NR4A3 induction, and their activity as assessed by the NBRE-LUC reporter assay. These results show that A_{2A}AR modulatory's effect on the ERK1/2-NR4A signaling axis is not limited to adenosinergic signaling.

Taken together, the results presented in this study establish a novel effector signaling axis downstream of adenosine, and suggest NR4A antagonism as a mechanism mediating A_{2A}AR anti-inflammatory effects in mast cells. Thus, this data contributes to the understanding of how receptor-specific signals are integrated towards modulation of the inflammatory response, which could facilitate the development of AR-based strategies of immunomodulation.

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5 Adenosine receptor modulation: Potential implications in veterinary medicine

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Review

Adenosine receptor modulation: Potential implications in veterinary medicine

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Abstract

Adenosine is a purine nucleoside whose concentration increases during inflammation and hypoxia and the many roles of this molecule are becoming better understood. Increased reactivity to adenosine of the airways of asthmatic but not of normal subjects underlines the role of adenosine in airway inflammation. The identification and pharmacological characterisation of different adenosine receptors have stimulated the search for subtype-specific ligands able to modulate the effects of this molecule in a directed way. Several compounds of different chemical classes have been identified as having potential drawbacks, including side effects resulting from the broad distribution of the receptors across the organism, have prevented clinical application.

In this article, the effects of adenosine's different receptors and the intracellular signalling pathways are reviewed. The potential of adenosine receptor modulation as a therapeutic target for chronic airway inflammation is considered, taking equine recurrent airway disease and feline asthma as examples of naturally occurring airway obstructive diseases. Other potential applications for adenosine receptor modulation are also discussed. As the intrinsic molecular events of adenosine's mechanism of action become uncovered, new concrete therapeutic approaches will become available for the treatment of various conditions in veterinary medicine.

Keywords: Adenosine; Adenosine receptors; Signal transduction; Chronic airway inflammation; Veterinary inflammatory conditions

Introduction

Adenosine is an endogenous nucleoside consisting of the purine base adenine in glycosidic linkage with the sugar ribose. Adenosine is present at low concentrations in the extracellular space and its concentration is greatly increased under metabolically stressful conditions as a result of enzymatic cleavage of the nucleotide adenosine 5′-monophosphate (AMP) by the 5′-nucleotidase (Arch and Newsholme, 1978). For example, an increase in adenosine formation occurs during inflammation when a large number of infiltrating inflammatory cells compete for a limited oxygen supply (Fig. 1). Intracellular concentrations of adenosine are kept low principally by its conversion to AMP by the enzyme adenosine kinase, but it may also be degraded to inosine by adenosine deaminase (Trams and Brown, 1974).

In mammals, adenosine can be released as the result of hypoxia, tissue injury and acute or chronic inflammation reaching local concentrations of up to 30 μM – a 150-fold increase over basal levels (Van Belle et al., 1987). Increased adenosine is found in bronchoalveolar lavage (BAL) and exhaled breath condensate of human patients with asthma (Driver et al., 1993; Huszar et al., 2002). Intracellular production of adenosine is increased during these stressful conditions and then transported across the membranes where it exerts its activities upon binding to adenosine receptors (ARs) (Thorn and Jarvis, 1996). The nucleoside mediates tissue protection by different mechanisms including increased oxygen supply/demand, ischemic preconditioning and stimulation of angiogenesis, and also as a paracrine inhibitor of inflammation with effects in the lung, heart and brain.

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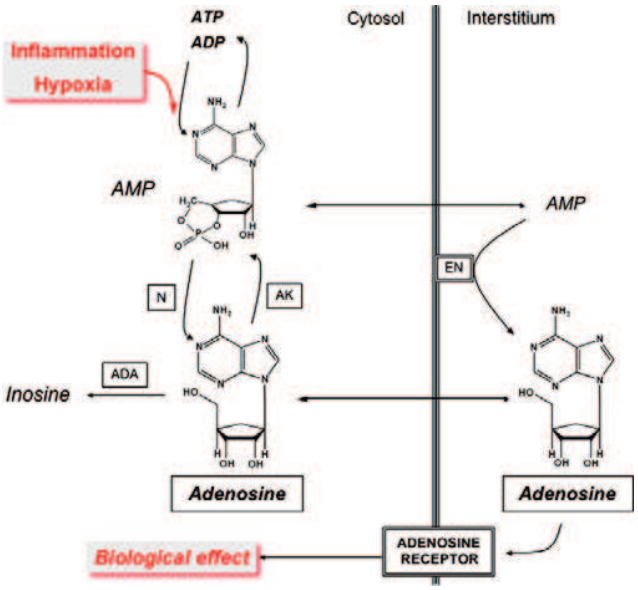

Fig. 1. Cycle of adenosine synthesis and degradation. Adenosine is formed from catalytic cleavage of cyclic AMP by *endo*- (N) or *ecto*-nucleotidases (EN). Once in the interstitium, adenosine binds and activates adenosine receptors, resulting biological effects. Adenosine is degraded by adenosine deaminase (AD) to inosine or it is recycled to AMP by adenosine kinase. Inflammation and hypoxia increase AMP concentrations.

Adenosine and inflammation

Persistent inflammation is a central feature of many diseases and invariably involves the recruitment and activation of inflammatory cells that lead to structural changes in the affected organs. In diseases of the lung, for example, over 100 inflammatory mediators have been implicated, and the blocking of a single mediator is unlikely to be effective. In fact, antagonists for these molecules have so far proved less effective than drugs with a broad spectrum of anti-inflammatory effects, such as glucocorticoids (Leckie et al., 2000). In recent years, the involvement of several factors acting as global stimulators or inhibitors has become better understood, thus opening new avenues for drug discoveries. Adenosine is one such molecule with therapeutic potential as an inflammatory modulator. For this reason, there is growing interest in elucidating the mechanisms by which adenosine inhibits the immune system, since these inhibitory adenosine receptors and their downstream signalling pathways are promising targets for new anti-inflammatory therapy in veterinary medicine.

G-coupled adenosine receptors

Four types of adenosine receptors (ARs) have been identified: A_1, A_{2A}, A_{2B}, and A_3. ARs have the typical topological structure of G-protein-coupled receptors (GPCR) with a central core domain consisting of seven transmembrane helices connected by three intracellular and three extracellular loops (Fig. 2). ARs have been cloned and characterised in several experimental animals, but information about the receptors in some other species of veterinary importance is limited. For example, in the cat, the sequence of just a fragment of the A_3AR is known, while in the horse, only the A_{2A}AR and A_3AR have been cloned (Brandon et al., 2006a,b).

Receptors A_1AR, A_{2B}AR, and A_3AR are among the smallest members of the GPCR family and possess a similar number of amino acids composing their primary structure. For example, the canine homologues of A_1AR, A_{2B}AR, and A_3AR consist of 326, 332, and 314 amino acid residues, respectively. Conversely, the canine A_{2A}AR is composed of 412 amino acids. All cloned species homologues of the A_{2A}AR are of similar mass, and this relatively large size is manifested in the carboxyl-terminal tail of the receptor, which is much longer than that of the other AR subtypes. The human A_1AR and human A_3AR display ca. 49% overall sequence identity at the amino acid level, while the human A_{2A}AR and human A_{2B}AR are 45% identical.

The identification of discrete receptor regions, or even single amino acids that contribute to ligand recognition and are responsible for discerning between agonist and antagonist ligands, has been an area of extensive investigation. Both transmembrane and extracellular regions of ARs have been implicated as playing a role in the formation of the ligand-binding pocket and several amino acids that contribute to the ligand-binding properties have been identified via mutagenesis (for review, see Moro et al., 2006). Discovering ligand–receptor interactions at a molec-

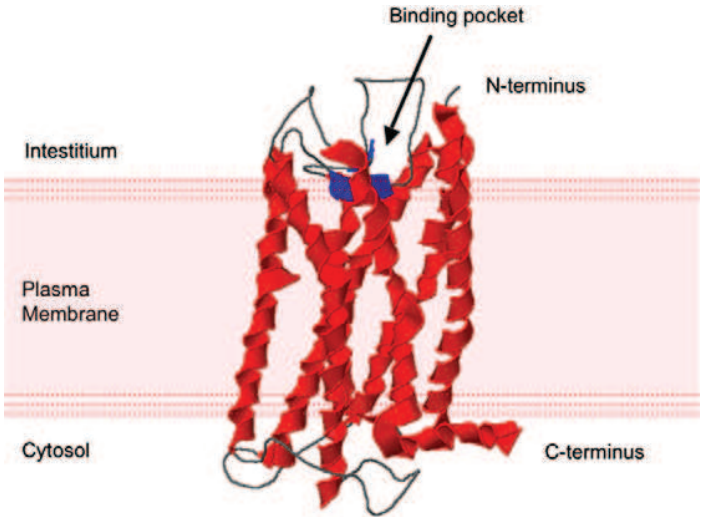

Fig. 2. Theoretical 3D-structure of the ARs. This model has been constructed using the high-resolution structure of rhodopsin as a template (Kim et al., 2003). The transmembrane helical domains are represented in red, while the intra- and extracellular loops are represented in grey. The adenosine-binding pocket is shown in blue. Note that the intracellular C-terminus end of the A_{2A}AR (not depicted) is composed of about 80–100 additional residues.

ular level will not only allow understanding the details of the process, but will also provide the basis for rational drug design.

Signal transduction and transcriptional effectors

Although the bioavailability of adenosine is an important determinant of its biological functions, the pattern of expression and distribution of ARs in the anatomical–structural sites of tissues and organs accounts for the observation that adenosine may exert either deleterious or protective roles. Adenosine receptors are expressed in a wide variety of tissues and each of the four subtypes so far has a unique pharmacological profile, tissue distribution and effector coupling. They exert their distinct effects by interacting with different G-proteins, which mediate activation or inhibition of the enzyme adenyl cyclase and therefore a change in the intracellular concentration of cAMP. In addition, other pathways, such as phospholipase C (PLC), Ca^{2+} and mitogen-activated protein kinases (MAPKs), are involved in adenosine signalling (Fig. 3).

The anti-inflammatory effects of the A_{2A} receptor appear to depend on its coupling to G_s (and to G_{olf} in striatum in the brain), stimulation of the adenylate cyclase and the consequent increase in intracellular cAMP. The A_{2B}AR couples both to G_s, promoting cAMP accumulation, and to G_q triggering Ca^{2+} mobilization and activation of PLC and MAPKs (Gao et al., 1999). Interestingly, cross-talk between G_s and G_q appears to regulate interleukin (IL)-4 production by A_{2B} in human mast cells (Ryzhov et al., 2006). The arachidonic acid pathway has also been implicated in A_{2B} signalling (Donoso et al., 2005). The A_1 and A_3ARs can inhibit the adenyl cyclase by coupling to G_i, but in addition they can activate K^+ and Ca^{2+} channels (Cronstein, 1994).

Interspecies differences in adenosine receptor-specific effects have been observed. For example, pharmacological differences between the rat and guinea pig A_{2B}AR and the canine adenosine A_{2B} receptor have been determined by using subtype-specific antagonists (Fozard et al., 2003). Species variations in the A_3AR binding properties of xanthine antagonists have also been identified (Salvatore et al., 1993). In this context, the A_{2A} receptor appears to be unique since, to date, all reports show that this receptor has anti-inflammatory effects, independently of the species.

Several publications have proposed that inhibition of NF-κB activity, a well-characterised pro-inflammatory transcription factor, is the direct target of cAMP-mediated AR anti-inflammatory effects. Genetic evidence in A_{2A}AR deficient mice indeed shows that an increase in pro-inflammatory cytokines at the transcriptional level in these animals is associated with enhanced NF-κB activity (Lukashev et al., 2004). In the absence of A_{2A}AR, adenosine can mediate a decrease in TNF-α independently of NF-κB, suggesting that adenosine's immunomodulatory effects depend on other transcriptional factors (Nemeth et al., 2003).

A recent report has suggested that another transcription factor, the nuclear factor of activated T cells (NFAT), can integrate signals originated from A_{2B} coupled to G_q and G_s and transduced by cAMP and IP_3 respectively (Ryzhov et al., 2006). Downstream components of the JNK activation pathway have been reported to be up-regulated in asthma (Demoly et al., 1995) along with enhanced

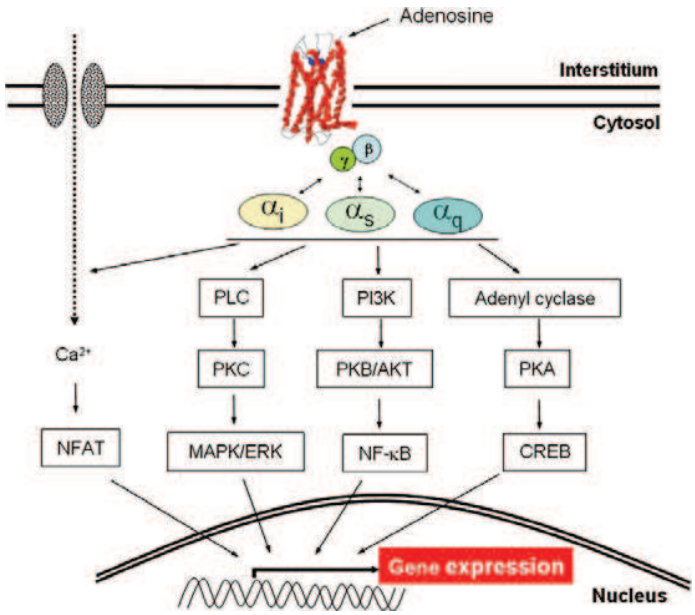

Fig. 3. Signal transduction pathways of ARs. Upon ligand binding, ARs couple to G-proteins and several signalling pathways become activated ultimately resulting in a finely tuned transcriptional response. α_i, α_s, α_q, β and γ are subunits of G-protein. PLC, phospholipase C; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphoinositide 3-kinases; PKB/AKT, protein kinase B; NF-κB, nuclear factor-κB; PKA, protein kinase A; CREB, cAMP response element binding.

NF-κB activation in both asthma and chronic obstructive pulmonary disease (COPD) (Hart et al., 1998; Di Stefano et al., 2002). Similarly, sustained NF-κB activity has been associated with recurrent airway disease in horses (see below). Therefore, inhibition of MAPK and NF-κB appear to be suitable molecular targets for chronic airway inflammatory conditions. The architecture of signal transduction is represented in Fig. 3.

The complexity of these findings illustrate the highly intricate network of adenosine signal transduction, which we are only starting to elucidate, and points to the need to systematically address the role of each player implicated in adenosine signalling.

AR agonists and antagonists

The search for potent and selective adenosine receptor ligands has been one of the most highly investigated areas in recent medicinal chemistry. While several analogues have been derived from adenosine itself (by modifications both at the adenine and ribose moieties), other more selective agonists have been obtained by empirical and semi-rational molecular modelling approaches. These strategies have brought up a large number of chemicals with differential affinity for the AR subtypes.

Interestingly, the efficacy at A_3AR is more easily diminished by structural modification than it is at the other subtypes and, therefore, adenosine derivatives have provided an alternative approach to the design of A_3 antagonists. For example, compounds substituted with large groups such as CCPA display full agonist effect at A_1 but behave as a moderately potent A_3 antagonist (Gao and Jacobson, 2002). Agonists for the A_{2B} receptor have remained elusive but recently, compounds with an improved selectivity profile for the human A_{2B}AR have been reported (Beukers et al., 2004).

Conversely, xanthines represented the starting point for the discovery of potent and more selective AR antagonists. In addition, screening chemical libraries allowed the identification of non-xanthine polyheterocyclic derivatives with AR antagonist properties. These include an impressively wide range of dihydropyridines, flavinoids, thiazoles and imidazoline derivatives among others. Xanthines are non-selective AR blockers, with theophylline and caffeine displaying micro molar affinities for A_1-, A_{2A}- and A_{2B}ARs; affinity for the A_3AR is lower, in the high micro molar range (Zhou et al., 1992). A major physico-chemical limitation of xanthines is their low water solubility and great emphasis has been placed in improving this property. As a result, several xanthine derivatives with improved water solubility have been successfully designed.

The effects of various AR ligands have been characterised in different animal species. Table 1 summarises the most relevant findings grouped by receptor type and compound. Notably, pronounced interspecies differences in the pharmacology of both AR agonists and antagonists have

Table 1
Adenosine receptor agonists and antagonists, whose pharmacological characteristics have been tested in animal studies

Class	Compound	Species, organ	Pharmacological effect	Remarks	Reference
Agonists					
A_1	CCPA	Cat, regional vascular bed	Vasodilatation		Bivalacqua et al. (2002)
	CPA	Cow, corneal endothelium	Increased cAMP and endothelial fluid transport		Tan-Allen et al. (2005)
A_{2A}	NECA	Horse, digital veins	Vasodilatation		Elliott and Brady (1998)
		Dog, mast cells	Degranulation	Proposed to act through A_{2B}AR activation	Auchampach et al. (1997)
	CGS21680	Equine receptor, heterologous expression in HEK cells	Inflammation down-regulation		Brandon et al. (2006a)
		Dog, exocrine pancreas	Water and bicarbonate secretion		Iwatsuki (2000)
	CVT-3146	Dog, myocardium	Vasodilatation	No renal vasoconstriction	Zhao et al. (2003)
	ALT-146e	Dog, myocardium	Reduction of infarct size in a canine model of myocardial infarction		Glover et al. (2007)
A_{2B}	LUF5835	N/A	N/A		–
A_3	IB-MECA	Equine, heterologous expression in HEK cells	Inflammation down-regulation		Brandon et al. (2006b)
		Cat, lung arteries	Reduction of ischemic injury		Rivo et al. (2004)
		Dog, myocardium	Reduction of infarct size after ischemic injury by preconditioning		Auchampach et al. (2003)
Antagonists					
A_1	DPCPX	Horse, forebrain	N/A		Chou and Vickroy (2003)
		Cat, pulmonary vascular bed	Inhibition of vasoconstrictor responses of adenosine at low vascular tone		Neely and Matot (1996)
		Feline, oesophageal smooth muscle cells	Inhibition of adenosine-mediated contraction		Shim et al. (2002)
		Sheep, respiratory system	Increased ventilation in normoxic conditions		Koos et al. (2005)
	CPX, BG9928	Dog, myocardium	Reduction of infarct size in a canine model of myocardial infarction		Auchampach et al. (2004)
	KW3902	Dog, kidney	Prevention of ischemia-reperfusion injury (A_1-mediated)		Li et al. (2005)
			Diuresis and natriuresis		Kobayashi et al. (1993)
A_{2A}	ZM241385	Horse, striatum (forebrain)	N/A		Chou and Vickroy (2003)
		Equine receptor, heterologous expression in HEK cells	Perpetuation of inflammation		Brandon et al. (2006a)
		Sheep, respiratory system	Inhibition of hypoxic ventilatory adaptive response		Koos et al. (2005)
		Cat and dog, cardiovascular	Reversion of adenosine-mediated blood pressure and vasodilatory effects		Poucher et al. (1996)
A_{2B}	8-SPT, XAC, CGS15943, CGH2473	Dog, saphenous vein smooth muscle	Inhibition of NECA-induced muscle contraction (an AR-agonist)	NECA response in this model appears to be A_{2B}- mediated	Fozard et al. (2003)
A_3	MRS1191	Cat, lung arteries	Prevention of AR-mediated protection of reperfused lung		Rivo et al. (2004)

N/A: no information available.
Abbreviations: ALT-146e: four-{3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxytetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid methyl ester; BG9928: (3-[4-(2,6-dioxo-1,2-dipropyl-2,3,6,7- tetrahydro-1H-purin-8-yl)-bicyclo [2,2,2]oct-1-yl] propionic acid; CPA: N6-cyclopentyladenosine; CCPA: 2-chloro-N6-cyclopentyladenosine; CGH2473: *N*-[4-(3,4-dichloro-phenyl)-5-pyridin-4-yl-thiazol-2-yl]-acetamide; CGS15943: 9-chloro-2-(2-furyl[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine; CGS21680: 2-[4-[(2-carboxyethyl)phenyl]ethylamino]-5-*N*-ethylcarboxamidoadenosine; CPX: 8-cyclopentyl-1,3-dipropyl-xanthine; CVT-3146: (1-{9-[(4*S*,2*R*,3*R*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-6-aminopurin-2-yl}pyrazol-4-yl)-*N*-methylcarboxamide; DPCPX: 8-cyclopentyl-1,3,dipropylxanthine; IB-MECA: N(6)-(3-iodobenzyl)-adenosine-5′-*N*-methylcarboxamide; KW3902: 1, 3-dipropyl-8-(3-noradamantyl)xanthine; LUF5835: 2-amino-4-(4-hydroxyphenyl)-6-(1H-imidazol-2-ylmethylsulfanyl)pyridine-3,5-dicarbonitrile; MRS1191: 3-ethyl-5-benzyl-2-methyl-4-phenylethynykyk-6-phenyl-1,4-(±)-dihydropyridine-3,5 dicraboxylate; NECA: 5′-(*N*-ethylcarboxamido) adenosine; XAC: xanthine amine congener; ZM241385: 4-(2-[7-amino-2-(2-furyl)[1,2,4] triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol; 8-SPT: 8-sulphophenyltheophylline.

been observed. The xanthine derivative DPCPX, for example, is highly potent and about 500-fold more selective for A_1 compared with the A_{2A} in a rat model (Lohse et al., 1987), whereas in humans this compound shows higher affinity towards the A_{2A} and the A_{2B} receptors.

A study comparing the A_{2B}-mediated muscle relaxant effect of AR antagonists of different classes showed similar affinities for the rat and guinea pig receptor, while for some compounds a weaker affinity for the dog receptor was recorded (Fozard et al., 2003). In the case of the sheep and human A_3AR, they bind xanthines with intermediate affinity, while the rat A_3AR shows much lower affinity for these compounds (Yang et al., 2005). These findings indicate that important species-dependent responses should be expected and such pharmacological heterogeneity should be taken into consideration at the different phases of compound development.

Adenosine in the lung: an inflammatory mediator with differential biological effects

There is growing evidence that adenosine plays an important role in respiratory disorders associated with lung inflammation. Adenosine (but not its metabolite inosine or the unrelated nucleoside guanosine) administered by inhalation was shown to be a powerful bronchoconstrictor of asthmatic but, importantly, not of normal airways (Cushley et al., 1983). Similarly, a recent study has established that the administration of adenosine 5′-monophosphate provoked airflow limitation in dogs with cadmium chloride-induced airway inflammation but not on healthy individuals (Hirt et al., 2007). Increased formation of adenosine occurs in chronically inflamed airways as demonstrated by elevated levels of adenosine both in BAL fluid and exhaled breath condensate of human patients with asthma with elevations correlating with the degree of inflammatory insult (Driver et al., 1993; Huszar et al., 2002).

Several findings have suggested that this nucleoside contributes as a mediator of bronchoconstriction in asthma. First, it was shown that adenosine is released into the circulation following allergen challenge of sensitised individuals (Holgate et al., 1980). Second, blockade of adenosine re-uptake by dipyridamole increased the bronchoconstrictor response to adenosine in asthma indicating that accumulation of extracellular adenosine was closely associated with the asthmatic airway response (Cushley et al., 1985). This has led to the current hypothesis that release of histamine and other spasmogens from resident airway mast cells may account for the bronchoconstrictor response to inhaled adenosine in asthma (Spicuzza and Polosa, 2003). Nevertheless, the role of histamine in similar pathologies in other species (i.e. recurrent airway obstruction in horses and feline asthma, see below) is less clear with antihistaminic agents showing erratic therapeutic effects.

Lung mast cells are known to be critical effector cells in the mediation of asthma and adenosine can influence their function. In vitro studies confirmed that adenosine and A_2 receptor analogues (e.g. 5′-*N*-ethylcarboxamideadenosine, NECA) could augment IgE-dependent mediator release from human mast cells (Church et al., 1982; Forsythe et al., 1999). More recently it has been shown that adenosine also upregulates Th2 cytokines in mast cells promoting IgE synthesis by B lymphocytes, suggesting that adenosine not only participates with mast-cell derived mediators during bronchoconstriction but that it also contributes to the maintenance of inflammation (Ryzhov et al., 2004).

Even though the mechanism by which cyclic nucleotides regulate the function of mast cells is not well understood, and that different subtypes of receptor seem to be involved in different species, these findings postulate AR ligands as promising therapeutic agents for the treatment of allergic asthma and other IgE-mediated diseases.

Adenosine receptors as targets in pulmonary disorders

Adenosine has long been recognised to influence the function of different cell types through activation of ARs. The high affinity A_1AR has been implicated in both pro- and anti-inflammatory aspects of disease processes. In the lung, A_1AR activation has been implicated in the regulation of bronchoconstriction in allergic rabbit models (Ali et al., 1994), suggesting a provocative role for A_1AR signalling in acute phases of asthma. Similarly, A_1AR antagonism has been shown to be beneficial in attenuating ischemia reperfusion and endotoxin-induced lung injury (Neely and Keith, 1995; Neely et al., 1997). However, the genetic removal of A_1AR from adenosine deaminase-deficient mice cause enhanced pulmonary inflammation along with increased mucus metaplasia and alveolar destruction, implicating a protective role for adenosine (Sun et al., 2005). Further studies need to be conducted to reconcile these apparently contradictory findings.

Signalling by A_{2A} is unequivocally linked to anti-inflammatory effects in the lung. For example, selective activation of the A_{2A}AR by the compound CGS-21680 produces broad-spectrum anti-inflammatory activity in a rat model of allergic asthma, suggesting that agonists could be useful in the treatment of this disease (Fozard et al., 2002). Also, CGS-21680 is able to protect the lung of Sprague–Dawley rats against shock-induced injury, implicating a novel therapeutic approach in preventing organ injury following trauma/hemorrhagic shock (Hasko et al., 2006).

Several lines of evidence suggest that the A_{2B}AR is deeply involved in the mechanisms by which adenosine induces bronchoconstriction and airway inflammation in asthma. Human mast cells have been shown to respond to A_{2B}AR stimulation by producing and releasing cytokines (Feoktistov and Biaggioni, 1995; Ryzhov et al., 2004). Also, pharmacological in vitro studies have shown that A_{2B} receptor antagonists can effectively inhibit adenosine-induced activation and degranulation of mast cells (Feoktistov et al., 2001). In light of these observations, the

development of selective adenosine A_{2B} antagonists has considerable therapeutic potential.

In the case of the A_3AR receptor, interspecies variation appears to play an important role. While abundantly expressed in rodent mast cells, apparently mediating degranulation in the rat and guinea pig (Zhong et al., 2003), A_3 receptors are found on human eosinophils, neutrophils and monocytes, where they seem to inhibit degranulation (Ezeamuzie and Philips, 1999; Gessi et al., 2002). Interestingly, IB-MECA, an A_3 agonist, has been able to attenuate the indices of injury and apoptosis following lung reperfusion in an isolated cat lung model. Furthermore, these effects could be blocked by the administration of MRS1191, a dyhydropyrimidine that is a selective A_3 antagonist (Rivo et al., 2004). On the other hand, it has been shown that the canine A_3 AR is structurally and pharmacologically more similar to the human than to the rat receptor (Auchampach et al., 1997).

Naturally occurring recurrent airway obstruction in animals: therapeutic potential of AR modulation in veterinary medicine

Equine recurrent airway obstruction (*RAO, previously known as COPD*)*:* Recurrent airway obstruction (RAO), also known as heaves, shares pathophysiological features with chronic asthma in humans, characterised by bronchospasm, mucus secretion, airway hyper-reactivity, and airway wall thickening together with deterioration of lung function (Robinson et al., 1996; Tsoumakidou et al., 2004). The major difference between these two diseases is that in heaves neutrophils dominate BAL fluids, as opposed as eosinophils in asthma. Even so, mast cells, macrophages and lymphocytes can be recovered by BAL from RAO-affected horses (Rush et al., 1998; Lavoie et al., 2001).

Several findings have established a close parallel between the two pathologies. First, histamine has been proposed as the main vasoactive mediator in this disease, with concentrations in BAL correlating with the numbers of mast cells and/or basophils. In addition, horses with a history of heaves have been found to be hyper-reactive to aerosol histamine during acute exacerbations (Derksen et al., 1985). Other autacoids including serotonin, leukotrienes, thromboxane and 15-HETE have also been implicated (McGorum et al., 1993; Franchini et al., 1998), although the role of these mediators in RAO can be questioned because neither antihistamine nor non-steroidal anti-inflammatory treatments improve lung function. Second, allergen-specific IgE and IgG levels can be measured in horses with heaves, suggesting that hypersensitivity may be the cause of RAO, as occurs in human and feline asthma (Eder et al., 2000; Ainsworth et al., 2002). In fact, elevated mRNA levels of IL-4, IL-5 but decreased levels of interferon (IFN)-γ have in measured in BAL-recovered cells from heaves-affected horses (Lavoie et al., 2001), suggesting a seemingly polarised T-helper 2 cytokine profile and therefore an allergic condition with similarity to asthma.

Other cytokines with altered profiles in RAO include IL-1β, tissue necrosis factor (TNF)-α and IL-8 (Giguere et al., 2002; Ainsworth et al., 2003), and IL-17 (Debrue et al., 2005). Finally, the downstream effects triggered by these cytokine profiles are driven by common transcription factors. For example, IL-1β and TNF-α appear to be responsible for the persistent pro-inflammatory NF-κB activity in bronchi of horses with RAO – a characteristic finding in asthmatic airways (Bureau et al., 2000). Other transcription factors that have been associated with asthma and RAO are AP-1, GATA-3 and CREB (Adcock, 1997; Nguyen et al., 2003; Couetil et al., 2006).

In view of the similarities between heaves and asthma, it could be hypothesised that adenosine plays a comparable role in the pathogenesis of both diseases. One early report showed that adenosine influences contractility in healthy equine tracheal smooth muscle (Norris and Eyre, 1982). To the best of our knowledge, no study has focused on the role of adenosine in the physiopathology RAO nor has the potential of AR modulation been assessed in this condition.

Recently, equine A_{2A}AR has been cloned in a heterologous system, where it inhibited NF-κB's activity in a concentration dependent fashion (Brandon et al., 2006a). The availability of these and other molecular biology tools will allow the development and validation of AR ligands with therapeutic potential for RAO in the horse.

Feline asthma

Feline asthma is one of the most common diseases of the lower respiratory tract in cats and has an extraordinary resemblance to human asthma. It involves bronchoconstriction, early and late allergen-induced airway responses, eosinophilia, airway wall remodelling and chronic inflammation, and it has been considered an ideal model of human asthma. Interestingly, airway inflammation can occur even in symptom-free asthmatic cats, indicating the need of appropriate therapy directed toward decreasing the underlying chronic inflammatory component of the disease (Padrid, 2000).

Studies on experimental models of asthma in cats have contributed to understanding the immunological events occurring during the spontaneously developing disease in the cat. Employing a Bermuda grass sensitization model, Norris Reinero et al. (2004) identified a T helper type 2 cytokine profile in BAL cells, together with airway eosinophilia and allergen-specific IgE, IgG and IgA both in serum and in BAL, with resemblance to both the findings in human and equine RAO (with the exception of the eosinophilia). Studies in *Ascaris suum*-sensitised cats revealed that serotonin (and not histamine) released upon mast cell degranulation appears to be the main autacoid involved in bronchoconstriction (Padrid et al., 1995), explaining the unpredictable effects of inhaled histamine on cats

affected with feline asthma. A recent study using this model determined a significant increase of enhanced pause (*Penh*, an index of bronchoconstriction) accompanied with radiographic and bronchoscopic alterations indicative of active remodelling together with elevated F_2-isoprostane concentration and MMP-9 activity in BALF (Kirschvink et al., 2007). The later two factors have been proposed as biological markers of lung inflammation and tissue remodelling and are also elevated in human asthma and COPD (Atkinson and Senior, 2003; Morrow and Roberts, 2002). Remarkably, A_{2A}AR activation inhibits MMP-9 secretion by neutrophils (Ernens et al., 2006).

Therefore, although further studies in cats (both on spontaneously occurring and on experimental animals) are clearly needed to confirm the involvement of adenosine in the pathophysiology in these species, the overall similarities with human asthma suggest that AR modulation could represent an attractive option for the treatment of asthma in this species.

Adenosine receptors modulation: more than airway inflammation

Beside their effects in cells of the immune system, ARs have been postulated as pharmacological targets for the treatment of several diseases. Some applications with potential in veterinary medicine are listed below.

Acute endotoxemia

Endotoxin plays a pivotal role in many pathophysiological states and diseases, and their recognition by cells of the immune system through Toll-like receptors results in downstream activation of NF-κB and MAPK pathways. In turn, pro-inflammatory cytokines are expressed, resulting in a coordinated biological response. During endotoxemia, the expression and release of cytokines (e.g. TNF-α) becomes deregulated, resulting in an uncontrolled inflammatory loop. The role of the A_{2A} receptor was first observed in A_{2A}AR knock-out mice, which showed an exacerbated inflammatory phenotype upon administration of an endotoxic stimulus (aseptic), uncovering a non-redundant mechanism for the down-regulation of inflammation (Ohta and Sitkovsky, 2001). A recent study has established that A_{2A}AR agonists can selectively inhibit the production of H_2O_2 in polymorphonuclear leukocytes isolated from septic shock patients (Kaufmann et al., 2007), underscoring the potential of this approach for the treatment of systemic inflammatory states.

Modulation of the pro-inflammatory activity of NF-κB appears to be the main downstream effect of A_{2A}AR. Recently, Brandon et al. (2006a) cloned and expressed the equine A_{2A}AR in a heterologous system and characterised its affinity to AR ligands. Employing a reporter gene assay, they showed that activation of the equine A_{2A}AR with the specific agonist CGS21680 reduces NF-κB transcriptional activity in a concentration-dependent manner. This pioneer work constitutes the base for future studies to examine the potential of A_{2A}AR modulation in the treatment of systemic inflammation in horses.

Ischemia–reperfusion injury

One of the most intensively investigated effects of adenosine is its protective action on reperfusion injury after ischemia. Several studies have shown the potential of targeting ARs for myocardial infarction treatment, as well as for lung and hepatic reperfusion injury. Different ARs appear to be involved in this response and accumulating evidence suggests that hepatic reperfusion injury is triggered by lymphocyte activation of A_{2A}Rs on bone marrow-derived cells (Day et al., 2004, 2005). A recent study has shown that initiation of the reperfusion-induced inflammatory cascade is dependent on IFN-γ production by NKT cells and that A_{2A} agonists can decrease the production of IFN-γ in this cell type (Lappas et al., 2006).

Ischemic preconditioning by adenosine could potentially ameliorate the effects of reperfusion injury in liver, pancreas, intestine and myocardium in diseases such as gastric dilatation and volvulus (GDV) in dogs, arterial thromboembolism in cats or gastrointestinal ischemic injury in horses. In dogs, for example, the A_3 receptor agonist IB-MECA has proven effective in reducing infarction size in an in vivo model of myocardial ischemia (Auchampach et al., 2003). Myocardial ischemia and reperfusion injury can lead to arrhythmia during GDV (Buber et al., 2007), indicating that ischemic preconditioning by adenosine receptor ligands could have a direct application in veterinary medicine.

Interestingly, adenosine could have additional pharmacological advantages in the heart of dogs with GDV by improving myocardial perfusion, and also by its direct antiarrhythmic effects (see below). Whether these experimental findings will translate into useful therapies for the treatment of pathological states associated with ischemia and reperfusion in veterinary medicine remains to be seen.

Arthritis

The A_3AR agonist IB-MECA has been shown to downregulate inflammation in a murine arthritis model after oral administration and to ameliorate the clinical and histopathological manifestations of adjuvant-induced arthritis in rats, in which A_3ARs were highly expressed (Fishman et al., 2006; Rath-Wolfson et al., 2006). Overexpression of A_3ARs was also detected in peripheral blood mononuclear cells of human patients with rheumatoid arthritis and receptor upregulation appears to be induced by inflammatory cytokines controlling the expression of the transcription factor NF-κB (Madi et al., 2007). Even though no information is at this point available implicating a role for adenosine (and A_3ARs) in immunomediated arthropathies in veterinary medicine, these recent findings may

reveal new and exciting approaches for the treatment of these pathologies.

Vasodilatation

Adenosine is a potent vasodilator in most vascular beds, except in renal afferent arterioles and hepatic veins where it produces vasoconstriction. Adenosine (as Krenosin) has been indicated for myocardial perfusion scintigraphy in humans. The A_{2A}AR has been shown to be involved in vasodilatation in the aorta and coronary artery. In fact, the A_{2A}AR agonist CVT-3146 has been proved a selective coronary vasodilator in dogs with minimal systemic haemodynamic effects, making it an attractive alternative for myocardial perfusion imaging (Zhao et al., 2003).

ARs as targets in renal disorders

Several lines of evidence support a protective effect of A_1AR activation in the kidney (for a recent review, see Vallon et al., 2006). For example, mice lacking A_1ARs show a completely blocked renal glomerular filtration rate, as well as increased renal injury after ischemia and reperfusion (Sun et al., 2001; Lee et al., 2004). These findings provide the basis for the development of drugs with great potential for the treatment of renal failure in both human and veterinary medicine.

ARs and the CNS

The observation that caffeine (a classical AR antagonist) enhances awareness and other activities on the nervous system at lower doses than those required for blocking phosphodiesterases, has stimulated the investigation of the effects of AR modulation in the central nervous system. A_{2A}- and A_1ARs are the primary mediators of the behavioural stimulatory effects of caffeine. These two receptor subtypes have been identified in the forebrain tissue of horses and their pharmacological profiles have been evaluated (Chou and Vickroy, 2003). In this study, caffeine and related methylxanthines were shown to antagonise CNS actions of adenosine effectively.

Several AR antagonists are being tested for the treatment of various nervous system disorders in humans (some at advanced clinical trial stages) ranging from Parkinson's disease to dementia and sleep disorders. Whether these findings will translate into concrete therapies in veterinary medicine remains to be seen.

Arrhythmia

The antiarrhythmic effect of adenosine (as Adenocard, Fujisawa) has been exploited in human medicine for several years to restore the normal rhythm in patients with paroxysmal supraventricular tachycardia (Zablocki et al., 2004). In contrast, relatively high doses of adenosine have been ineffective in abolishing supraventricular tachycardia in dogs (Wright, 2000). Moreover, practical issues such as its short half-life and the need to administer it very rapidly through a central vein have limited its application in veterinary medicine.

The antiarrhythmic effect of adenosine is mediated by A_1AR activation, which reduces heart rate and atrial contractility and also attenuates the stimulatory action of catecholamines. Several A_1AR agonists are in advanced development stages for this application, without the side effects of other AR subtypes (i.e. hypotension). The availability of these selective compounds may result in improved pharmacological options for the treatment of selected arrhythmias in veterinary medicine.

The future of AR modulation in veterinary medicine

With the availability of pharmacological probes and knockout models for most of the four AR subtypes, the implications of adenosine signalling are now been recognised. In the past, undesirable side effects associated with the wide tissue distribution of ARs have often prevented candidate drugs from performing successfully in clinical trials. This has remained a challenge for medicinal chemistry and we are now only starting to see compounds in advanced phases of development for the treatment of specific conditions.

Even if our understanding of the role of adenosine in veterinary medicine has lagged behind, the development of novel molecular biology research tools will allow us to assess the therapeutic potential of AR modulation in species of veterinary interest, primarily for the treatment of chronic airway inflammatory diseases but also for a myriad of other conditions.

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6 Association between increased adenosine concentration in bronchoalveolar lavage fluid and lower airway inflammation in horses

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Conceived the project and recruited the necessary funds; established the working group; designed and supervised the experimental work; contributed to the collection and processing of BAL samples; analysed the data; wrote the manuscript. Corresponding author.

Manuscript

Association between increased adenosine concentration in bronchoalveolar lavage fluid and lower airway inflammation in horses

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Abstract

Several lines of evidence showing the involvement of adenosine in the pathogenesis of chronic airway conditions have led to new therapeutic strategies to limit airway inflammation. In this study, detectable levels of adenosine in bronchoalveolar lavage (BAL) samples from 11 horses with non-infectious lower-airway inflammation and 14 healthy controls are reported, with significantly higher values in airway-compromised horses. Although these increased levels did not correlate with changes in neutrophil percentage in BAL fluid, a positive association between adenosine levels and signs of lower airway inflammation (clinical score) was observed. These findings suggest, first, that adenosine could be considered a marker of equine airway inflammation with diagnostic value and, second, that this nucleoside plays a role in respiratory disease in this species, advocating for the development of new strategies for the treatment of equine lower airway inflammation based on the regulation of this signalling axis.

Keywords: horse; airway inflammation; adenosine; bronchoalveolar lavage

In recent years, the role of adenosine signalling in airway inflammation has become better understood. This endogenous nucleoside is a metabolic by-product that accumulates in BAL and exhaled breath condensate in patients with asthma (Driver et al., 1993; Huszar et al., 2002). Inhalatory administration of adenosine induces bronchoconstriction of asthmatic but not of normal airways, and in addition to this effect, adenosine modulates the course of inflammation through the release of cytokines and chemokines from various cell types in the airways (Caruso et al., 2009). Therefore, strategies based on adenosine receptor ligands are currently being developed aimed at controlling adenosine-mediated inflammatory signalling (Dip, 2009).

Persistent inflammation is a central feature of chronic non-infectious airway conditions in the horse such as recurrent airway obstruction (RAO) and inflammatory airway disease (IAD) (Robinson, 2001). Therefore, we wanted to ascertain whether adenosine signalling is associated with inflammation in the equine lower airways. 11 adult horses (3 mares and 9 geldings; age range 7–17 years, median= 9.5 years) that were presented with mild to moderate signs of lower airway inflammation at the Equine Clinic at the University of Zurich were included in the study. Symptoms included exercise intolerance, coughing, nasal discharge and were diagnosed with lower airway inflammatory disease (either IAD or RAO) on the basis of history, clinical findings, mucus and cytological examination of BAL samples. Animals that were medicated less than 28 days prior to the examination as well as those with concomitant signs of infectious or systemic disease were excluded from the study. In addition, 14 healthy controls (4 mares and 10 geldings; age range 5–22 years, median= 11 years) with no history of airway inflammation or clinical signs of respiratory disease and normal BAL cytology were employed as a control group. Each horse was clinically examined and given a weighted score, using a scale with

range 0 to 25 as described by (Tesarowski et al., 1996). All clinical observations were done by 2 individuals, which were blinded to the group identity of the horses.

Bronchoalveolar lavage was performed as described (Zhang et al., 2010). For cytological analysis, cytospin slides were prepared, stained with May-Grünwald Giemsa and at least 400 cells from each sample were counted. In addition, BAL-samples were collected in vials containing 0.1μM iodotubericidin and 10μM of the erythro-9-(2-hydroxy-3-nonly)adenine (Sigma), centrifuged and cell-free fractions immediately frozen. All samples were analyzed by a specific spectrometry-based assay at a later time point (Ren et al., 2008). Urea concentration was determined both in BAL and serum samples obtained simultaneously (QuantiChrom assay, BioAssay Systems), and the ratio between these values was employed as an indicator of epithelial lining fluid dilution and employed for adenosine concentration normalization. BAL cytology, clinical score and normalized adenosine concentration are summarized in table 1.

Every BAL sample analysed revealed adenosine levels clearly above the limit of detection (LOD: 0.02 pg/mL BAL). Furthermore, adenosine concentration was significantly higher in airway-compromised horses (range 26.4-377.3 pg/mL, median 112.8 pg/mL) as compared to healthy controls (range 17-191 pg/mL, median 43.6 pg/mL), suggesting a role for this nucleotide in the development of lower airway inflammation in this species (Fig. 1).

Because the determination of lower airway inflammation in the horse relies heavily on clinical findings and BAL cytology (in addition to medical history), we hypothesized that adenosine concentration could be associated to the degree inflammation when assessed by these criteria. Fig. 2A shows the relationship between adenosine concentration in BAL and clinical score for the horses studied. All healthy controls had a clinical score of 2 or lower (average: 0.6) and with the exception of one horse,

adenosine values were below 130 pg/mL. On the other hand, elevated clinical scores (average: 4.6) were related to higher adenosine concentrations in airway-compromised horses. Statistical analysis indeed revealed a positive correlation between adenosine concentration and clinical score.

The assessment of cytological alterations in BAL, in particular neutrophil count, remains a major diagnostic tool in equine airway disease (Couetil et al., 2001). In fact, the percentage of neutrophils serves as an indicator of the severity of the underlying inflammation. In this study, however, a correlation between adenosine concentration and neutrophil count could not be established (Fig. 2B). As a matter of fact, all but one of the samples with adenosine levels over 200 pg/mL showed marginal to moderate increments in neutrophil numbers, suggesting no association between these variables. Yet, this observation should be interpreted with care because only three samples with neutrophil count indicative of moderate to severe airway inflammation (more than 20%) were tested. Analysis of a larger set of samples with high percentage of neutrophils (i.e. acute RAO phase) would be required to clarify the relationship between these two parameters.

The presence of increased adenosine concentrations in airway-compromised horses would suggest that adenosine could represent a marker of inflammation. However, in view of the partial overlap in the values of both groups of horses analyzed, assessment of adenosine levels in BAL does not seem to be suitable as the sole indicator of disease, but should be interpreted in combination with other diagnostic parameters (i.e. clinical signs).

The data presented suggest the presence of an adenosine signaling axis in the equine airways. Two lines of evidence support this hypothesis. On one hand, an early report indicated that adenosine influences contractility in equine tracheal smooth muscle (Norris and Eyre, 1982). On the other, we recently showed enhanced

IL-6 transcriptional responses to adenosine receptor ligands in BAL cells from horses with lower airway inflammation (Zhang et al., 2010). Therefore, the high adenosine levels reported here suggest that this nucleoside not only contributes to bronchoconstriction during disease, but that it also acts as a pro-inflammatory mediator in the bronchoalveolar milieu of airway-compromised horses.

Altogether, our findings indicate that adenosine plays a role in equine respiratory disease and argue for the development of new strategies for the treatment of highly prevalent lower airway inflammatory conditions, such as RAO and IAD.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Table 1- BAL cytology, clinical score and adenosine concentration from healthy (1 to 14) and airway compromised horses (15-25).

Horse	Sex	Age	Neutrophils (%)	Lymphocytes (%)	Macrophages (%)	Eosinophils (%)	Basophils (%)	Clinical score	Adenosine concentration (pg/µL)
1	M	10	1.3	46.6	52.1	0.0	0.0	0	40.5
2	F	10	1.3	45.9	52.1	0.7	0.0	1	43.1
3	M	17	1.9	52.2	45.4	0.0	0.5	0	46.9
4	M	5	5.7	56.3	36.8	0.0	1.2	1	108.6
5	F	15	6.1	69.3	24.2	0.0	0.4	1	52.9
6	M	5	0.8	48.9	46.8	0.0	3.5	0	38.7
7	F	8	2.1	41.6	55.2	0.0	1.1	0	44.1
8	M	16	3.4	62.4	33.3	0.0	0.9	1	29.0
9	F	9	1.9	46.9	48.3	0.5	2.4	0	127.7
10	M	5	3.5	57.9	38.2	0.0	0.4	0	31.2
11	M	5	1.2	36.0	62.2	0.0	0.6	0	101.1
12	M	10	6.0	39.0	53.4	0.0	1.6	2	17.0
13	M	13	3.5	32.2	60.4	0.0	0.0	2	17.2
14	M	9	0.5	60.5	37.0	0.5	1.5	1	191.8
Average		**9.8**	**2.8**	**49.6**	**46**	**0.1**	**1**	**0.6**	**63.57**
15	M	6	5.7	61.9	31.5	0.0	1.0	1	85.4
16	M	12	5.6	38.1	53.8	0.2	2.5	8	215.8
17	M	7	10.4	53.9	35.7	0.0	0.7	4	26.4

18	M	7	7.3	61.4	31.3	0.1	1.0	0	57.8
19	F	15	4.1	25.2	67.4	0.0	0.5	5	34.7
20	M	5	4.5	46.8	47.8	0.0	0.2	4	377.3
21	F	11	31.9	20.2	43.7	0.5	0.0	3	112.8
22	F	13	12.4	59.7	27.2	0.0	0.7	8	316.3
23	M	8	16.0	48.1	31.4	0.0	4.5	8	201.7
24	M	22	76.3	11.8	11.4	0.1	0.5	8	352.0
25	M	13	50.0	23.9	25.7	0.0	0.4	2	103.5
Average		**10.8**	**20.7**	**41.1**	**37.1**	**0.1**	**1.1**	**4.6**	**171.23**

Figure legends

Fig. 1. Adenosine concentration in BAL fluid samples in airway-compromised and control horses. *: $P \leq 0.05$, between groups (Mann-Whitney *U*-test).

Fig. 2. A) Positive correlation between adenosine concentration and clinical score for all 25 horses included in the study (Spearman *r* correlation coefficient, $P \leq 0.05$; slope: 0.01823). B) Association between adenosine concentration and neutrophil count. No correlation was observed. (●): horses diagnosed with lower airway inflammation. (○): healthy controls.

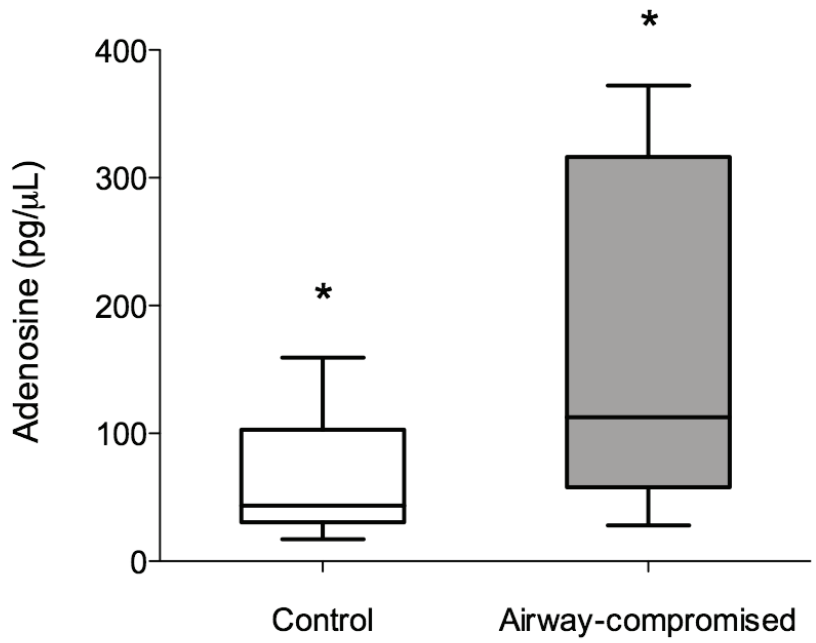
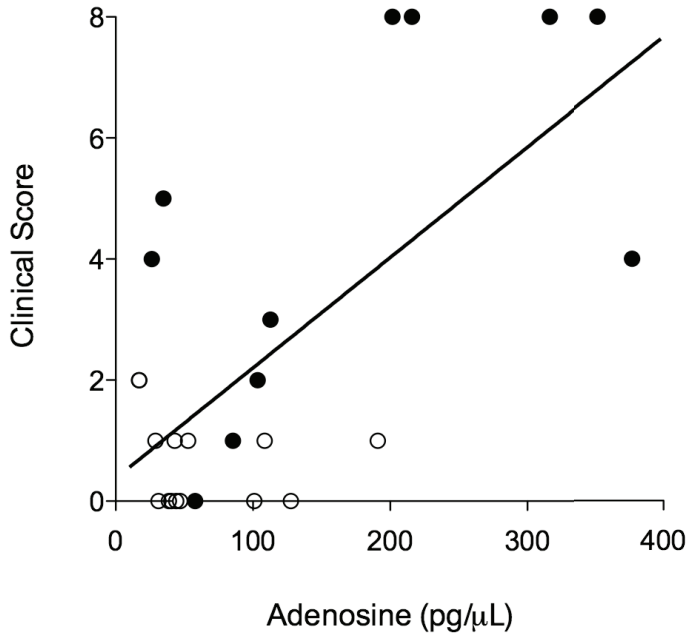

Adenosine (pg/μL)
400
300
200
100
0
*
*
Control
Airway-compromised

Fig 1

A


Clinical Score
8
6
4
2
0
0
100
200
300
400
Adenosine (pg/μL)

B

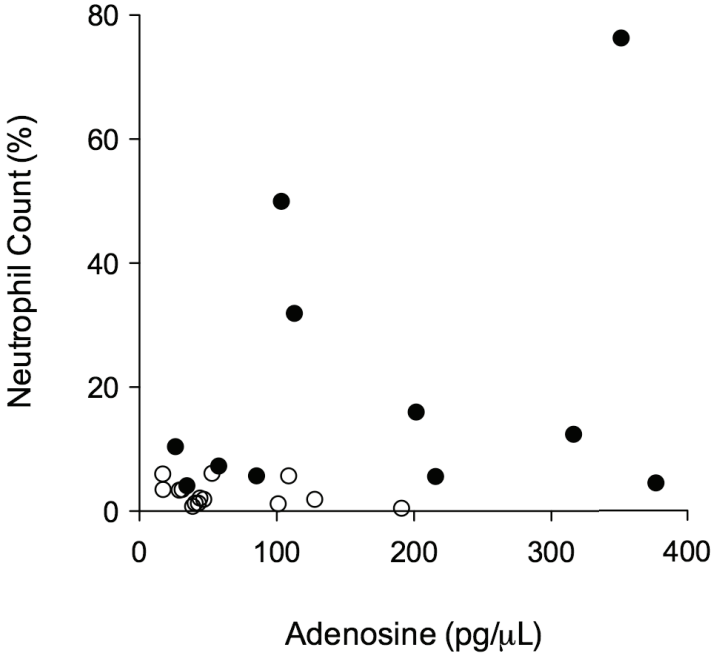

Neutrophil Count (%)
80
60
40
20
0
0
100
200
300
400
Adenosine (pg/μL)

Fig 2

7 Enhanced IL-6 transcriptional response to adenosine receptor ligands in horses with lower airway inflammation

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Contribution of Ramiro Dip

Conceived the project and recruited the necessary funds; established the working group; designed and supervised the experimental work; contributed to the collection and processing of BAL samples; analysed the data; wrote the manuscript. Corresponding author.

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Enhanced IL-6 transcriptional response to adenosine receptor ligands in horses with lower airway inflammation

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Keywords: horse; airway inflammation; adenosine; IL-6; adenosine receptor

Summary

Reasons for performing study: **Accumulation of extracellular adenosine has been closely associated with human asthmatic responses. However, the relevance of adenosine signalling in equine airways has not previously been investigated.**

Objectives: **To determine the expression of adenosine receptors (AR) in bronchoalveolar lavage (BAL) cells and assess the reactivity of these cells to AR ligands *ex vivo*, employing IL-6 as readout of adenosinergic inflammatory signalling.**

Methods: **Eight horses with varying degrees of lower airway inflammation and 10 healthy controls were analysed. Expression of AR-subtypes in each BAL sample was determined by quantitative RT-PCR and compared to that in 13 other tissues. Bronchoalveolar lavage cells were stimulated either with the adenosine analogue NECA, CGS-21680 (A_{2A}AR selective agonist) or with a combination of NECA and SCH-58261 (A_{2A}AR antagonist) and IL-6 expression assessed.**

Results: **Bronchoalveolar lavage cells predominantly expressed A_{2B}AR, with lower A_{2A}AR levels and marginal A_3AR expression; A_1AR was not detected. This pattern was similar to that of PBMCs but different from the other tissues tested. No significant differences in AR expression in BAL cells from both groups were detected, although a trend for decreased A_{2B}AR in airway-compromised horses was observed. Treatment of BAL cells with the nonselective agonist NECA upregulated IL-6 expression in cells from airway-compromised horses, but levels remained unchanged in control animals. Furthermore, blockage of A_{2A}AR with SCH-58261 enhanced IL-6 mRNA induction by NECA in both groups, with higher levels in airway-compromised horses; the amplitude of this response correlated with neutrophil count.**

Conclusions: **These results demonstrate the presence of an adenosine/IL-6 inflammatory axis in the bronchoalveolar milieu of airway-compromised horses. While A_{2B}AR is the predominant proinflammatory AR subtype expressed, A_{2A}AR appears to modulate inflammatory signalling (IL-6 expression) by adenosine.**

Potential relevance: **This study supports selective AR targeting as a potential therapeutic approach for the modulation of inflammation in the equine lower respiratory tract.**

Introduction

Chronic inflammatory airway diseases are remarkably prevalent in the horse. Recurrent airway obstruction (RAO) and inflammatory airway disease (IAD) are 2 such conditions that share a number of clinical, cytological and functional similarities. Recurrent airway obstruction is characterised by bronchospasm, mucus secretion, airway hyperreactivity and airway wall thickening together with deterioration of lung function and affects older individuals, while IAD-affected horses are usually younger with clinically milder signs (Couetil *et al*. 2007).

Adenosine is an endogenous byproduct of ATP metabolism normally present at low concentrations in the extracellular space. Its levels are greatly increased under metabolically stressful conditions such as tissue injury, hypoxia and acute or chronic inflammation, reaching local concentrations of up to 30 µmol/l, a 150-fold increase over basal levels (Van Belle *et al*. 1987). This nucleoside activates 4 types of G-coupled adenosine receptors (ARs), which are expressed on various inflammatory and stromal cells: A_1, A_{2A}, A_{2B} and A_3. ARs have well characterised anti-inflammatory and wound healing activities, demonstrating the importance of these signalling pathways in tissue protection (Hasko *et al*. 2008). In some situations, however, adenosine generation and AR activation have proinflammatory activities that amplify tissue injury (Blackburn 2003). These observations have driven research efforts to develop selective agonists or antagonists for AR subtypes for use in novel therapies for chronic inflammatory conditions.

There is growing evidence that adenosine plays an important role in respiratory disorders in the lower airways. Increased amounts of adenosine are found in bronchoalveolar lavage and exhaled breath condensate of human patients with asthma (Driver *et al*. 1993; Huszar *et al*. 2002) and, when administered by inhalation, adenosine was shown to be a powerful bronchoconstrictor in asthmatics but not in healthy subjects (Cushley *et al*. 1983). Furthermore, blockade of adenosine reuptake by dipyridamole increased the bronchoconstrictor response to adenosine in asthma indicating that its accumulation is closely associated with the asthmatic airway response (Cushley *et al*. 1985).

Recent studies have investigated the adenosine pathway in the horse. The affinity of several ligands to AR subtypes has been studied in horse neutrophils (Sun *et al*. 2007). Equine A_{2A} and

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A_3ARs have been cloned in this species (Brandon *et al*. 2006a,b). Furthermore, activation of A_{2A}AR in equine monocytes has been shown to inhibit lipopolysaccharide-induced TNF-α production, suggesting a therapeutic potential for AR modulation in inflammation (Sun *et al*. 2008a). However, the relevance of adenosine signalling on the regulation of inflammatory cytokines in the equine respiratory tract has not previously been examined.

Here, we employed an *ex vivo* approach to assess the role of adenosine in the physiopathology of lower airway inflammation in the horse. First, we established the expression profile of ARs in BAL cells and compared it to cerebellum, cortex, myocardium, aorta, spleen, stomach, jejunum, caecum, kidney, adrenal gland, eye, fat and peripheral blood mononuclear cells (PBMCs). Second, we determined that AR levels do not differ significantly between compromised and control animals. Third, we investigated the reactivity of BAL-recovered cells from horses with lower airway inflammation and healthy controls to AR ligands and determined that nonselective pharmacological activation of ARs leads to increased expression of the pro-inflammatory cytokine IL-6 in cells from horses with lower airway inflammation but not from control animals. Finally, we observed that selective blockage of A_{2A}AR further increases IL-6 transcription, leading to remarkably high levels in horses with lower airway inflammation. These data suggest that adenosine signalling is a relevant inflammatory mechanism during lung inflammatory conditions and that A_{2A}AR acts as an endogenous modulator of inflammatory signalling in the equine airway.

Materials and methods

Horses

Eight adult horses (2 mares and 6 geldings; age range 5–18 years, median = 13 years; weight range 450–516 kg) were presented with varying degrees of lower airway inflammation at the Equine Clinic at the University of Zurich. Clinical signs included exercise intolerance, coughing, nasal discharge and the horses were diagnosed with lower airway inflammatory disease (either IAD or RAO) on the basis of history, clinical findings, mucus and cytological examination of BAL samples.

Ten healthy horses with no history of airway inflammation or clinical signs of respiratory disease and normal BAL cytology were employed as a control group (7 mares and 3 geldings; age range 8–17 years, median = 9 years; weight range 480–605 kg). These horses belonged to the same stud, were vaccinated and dewormed regularly, stabled in the same barn and fed hay and grain twice a day. Sample collection of control horses was approved by the veterinary district authorities; anaylsis of samples obtained for diagnostic purposes did not require approval.

Bronchoalveolar lavage and differential cytology

Horses were sedated with a combination of xylazine (0.1 mg/kg bwt i.v.)[1] and butorphanol (0.02 mg/kg bwt i.v.)[2]. A fibroscope was introduced into a nostril, directed into the trachea and advanced until it wedged in a bronchus. During the advancement of the bronchoscope the bronchial surface was continuously anaesthetised with 50 ml of a solution containing 0.4% lidocaine and 0.9% sodium chloride injected through a sterile catheter passed through the biopsy channel. Two-hundred-and-fifty ml of prewarmed (37°C) sterile physiological saline solution were then infused into the bronchus and immediately reaspirated into a sterile glass flask kept on ice.

Bronchoalveolar fluid was centrifuged at 200 ***g*** for 10 min and rinsed x1 with PBS. A cytospin slide was prepared and stained with May-Grünwald Giemsa for cytological analysis. At least 400 cells from each lavage sample were counted.

Cell culture and ex vivo *stimulation*

The remaining cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% FBS[3], 0.1 u/ml penicillin and 0.1 mg/ml streptomycin[3] at a concentration of 2×10^6/ml, 10^6 cells were seeded in each well of a 24 well plate and cultured overnight at 37°C under humidified air containing 5% CO_2. The following day cells were treated for either 3 h with the AR ligands 5′-N-ethylcarboxamidoadenosine (NECA, 10 µmol/l), 2-p-(2-carboxyethyl)phen ethylamino-NECA (CGS-21680, 0.1 µmol/l) or the combination of NECA and 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine (SCH-58261, 1 µmol/l). All chemicals were from Sigma[4]. The use of lidocaine as a local anesthetic during the BAL procedure did not contribute to any observed effect as there was an extensive rest period before the cells were used.

RNA extraction and cDNA synthesis

Total RNA was isolated and cDNA synthesised as described (Franchini *et al*. 2000). Briefly, RNA was isolated using the total RNA isolation kit, which includes a DNAse treatment step[5]. RNA concentration was measured by optical density at 260 nm and RNA quality assessed by 260/280 absorvance ratio and cDNA synthesised with the cDNA reverse transcription kit[6] following the manufacturer's instructions. Briefly, 0.5 µg of total RNA was mixed with 1 µl of random primers, 2 µl of reverse transcription buffer and 0.8 µl dNTP mix, 1 µl multiScribe reverse transcriptase and 1 µl RNA inhibitor to a final volume of 20 µl. The mix was incubated at 25°C for 10 min and at 37°C for 120 min and then heated at 85°C for 5 s. The reactions were stored at -20°C until used.

Samples for adenosine receptor expression profiling

Immediate *post mortem* samples were obtained from one healthy horse. 200 mg of cerebellum, cortex, myocardium (left ventricle), aorta, spleen parenchyma, jejunum and caecum (submucosa and muscular), kidney (cortex), adrenal (cortex and medulla), eye (conjunctiva) and retroperitoneal fat were mechanically disrupted in a 3 ml Dounce homogeniser in lysis buffer[5]. Additionally, 10 ml of whole blood were obtained from this animal and PBMCs isolated employing a Ficoll reagent, following the manufacturer's instructions[7]. Ribonucleic acid from each tissue and PBMCs was extracted and cDNA synthesised as described above.

Selection of primers for expression analysis

The ARs primers for RT-PCR were designed based on the complete (A_{2A}), predicted (A_1, A_{2B}) or partial cd sequences (A_3) (GenBank accession numbers: NM_001081897, XM_001496410, XM_001918390 and AY_011243, respectively). Primers were selected for RT-PCR performance and absence of artifacts. The

TABLE 1: Primer sequences and expected product lengths of the genes analysed

Gene	GenBank accession No.	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product length (bp)
A_1AR	XM_001496410	TCAACATCGGGCCACGGACC	CAGGTTGTTCCAGCCAAACAGAG	242
A_{2A}AR	NM_001081897	CGCGAGTTCCGCCACACCTT	CCTGCTCTGCATCGCTGCCA	124
A_{2B}AR	XM_001918390	TCACDCAGAGCTCCATCTTC	CAAAGGCAAGGACCCAGAGG	141
A_3AR	AY_011243	AGGGACACAGGAAGCCAGCTCA	GGAAGCCCTGCAGCTTCTGTTCC	213
IL-6	NM_001082496	ATGAGTGGCTGAAGAACACAACAAC	AGGAATGCCCATGAACTACAACAAT	131
GAPDH	NM_001163856	TGGCATGGCCTTCCGTGTCC	GCCCTCCGATGCCTGCTTCAC	118

specificity of the amplified product was confirmed by sequencing. Amplification efficiency was calculated based on the CT values obtained from a serial dilution of a positive control cDNA (90–110%). Sequences for A_1, A_{2A}, A_{2B} and A_3-ARs, IL-6 and GAPDH primers and their expected product lengths are listed in Table 1. PCR reactions with these primers yielded single bands of the expected sizes. No unspecific secondary primer bands were observed (Supplementary Fig S1).

Quantitative real-time polymerase chain reaction (RT-PCR)

One µg cDNA was amplified in a 20 µl PCR reaction containing 10 µl of SYBR green supermix[8] and 10 µmol/l of forward and reverse primers. The PCR was performed in the iCycle IQ multicolour real time PCR detection system[8] with an initial incubation step at 95°C for 10 min to activate the enzyme and 45 cycles of amplification, including a denaturation at 95°C for 15 s and a 1 min extension at 60°C. Samples without cDNA were always included in the amplification reactions to check for contamination. All reactions were performed in triplicates. Fold changes in the expression of each gene was calculated using the 2-ΔΔCT method (Livak and Schmittgen 2001), with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript as an internal standard. Relative AR and IL-6 levels in resting cells are expressed as the ratio of the CT value for the gene of interest/CT for GAPDH. Mean GAPDH CT values for airway and control horses are 19.98 and 20.35 (difference not significant), with coefficients of variation are 3.68 and 3.65%, respectively. Finalised RT-PCR reactions were loaded in a 2% agarose gel containing 0.1 mg/ml etidium bromide for visualisation of DNA and subjected to electrophoresis.

Data analysis

Statistical analysis was performed using the PASW Statistics software[9]. Data were analysed by nonparametric tests (Mann-Whitney *U* test, Spearman correlation coefficient) or with the *t* test with Welch correction when values showed normal distribution (A_{2B}AR expression). Differences between groups were considered significant when P values were less than 0.05.

Results

Cytological characterisation of BAL samples

Control horses had cytological values within physiological range (neutrophils <5%, lymphocytes 30% to 60%, macrophages 40–70%, eosinophils <0.5%, basophils <2%), with the exception of 2 horses with a marginal increase of neutrophils and one horse with high basophil count.

On the contrary, airway-compromised horses showed significantly higher counts for nondegenerate neutrophils. Interestingly, 2 horses also had increased eosinophils (*Horses 15* and *16*, 6.3 and 2.8%, respectively), suggesting that this is a rather heterogeneous group with varied pathophysiological conditions and/or disease states. The cytological findings of the horses included in the study are summarised in Table 2.

Tissue-specific AR expression profiles

RT-PCR analysis from one healthy horse revealed a heterogeneous expression profile for the 4 AR subtypes across the tissues explored. The RT-PCR reactions were loaded on an agarose gel (Fig 1a) and analysed quantitatively (Fig 1b). Product length, specificity and the absence of primer-dimer products were confirmed in BAL cells and cerebellum (Supplementary Fig S1).

While A_1AR could not be detected in BAL–recovered cells, this AR was expressed both in cerebellum and cortex, jejunum, caecum and eye. Exceptionally high A_1AR levels were detected in fat, in agreement with previous reports showing that this subtype is heavily expressed in this tissue, mediating most of the effects of adenosine on adipocytes (Kaartinen *et al*. 1991). A_{2A}AR could be detected in all samples, although levels were very low in cerebellum, cortex and kidney. Significant levels of this AR were identified in spleen (where it was the predominant AR type), in adrenal and in myocardium, while high levels were detected in PBMCs. Among all ARs, the A_{2B} subtype was the most commonly detected. Expression in PBMCs was very prominent, with high levels in BAL cells and cerebellum, and intermediate in cortex, aorta and adrenal gland. A_{2B}AR transcripts were less abundant in myocardium, stomach, spleen, fat and eye. In contrast, expression of the A_3AR was less common, with highest levels in PBMCs and lower ones in BAL cells, jejunum, adrenal, fat and eye. Interestingly, the expression pattern in PBMCs was similar to that of BAL-cells, but with overall expression values about 10-fold higher than BAL.

AR expression in BAL cells from airway-compromised and healthy horses

Studies examining the expression of ARs in asthmatics and COPD patients support the hypothesis of a fundamental alteration in adenosine signalling in these patients (Varani *et al*. 2006). The analysis presented here revealed A_{2B}AR as the predominant subtype in equine BAL cells, with lower levels of A_{2A}AR and marginal A_3AR expression. Therefore, we wanted to assess the expression pattern of ARs in BAL from airway-compromised horses as compared to healthy controls. Although the differences in A_{2A}AR and A_{2B}AR expression between both groups were not significant, a tendency for decreased A_{2B}AR expression in airway-

TABLE 2: Cytological findings in BAL cells from healthy (1–10) and airway-compromised horses (11–18)

Horse	Sex	Age	Neutrophils (%)	Lymphocytes (%)	Macrophages (%)	Eosinophils (%)	Basophils (%)
1	F	10	1.3	45.9	52.1	0.7	0
2	M	17	1.9	52.2	45.4	0	0.5
3	M	5	5.7	56.3	36.8	0	1.2
4	F	15	6.1	69.3	24.2	0	0.4
5	M	5	0.8	48.9	46.8	0	3.5
6	F	8	2.1	41.6	55.2	0	1.1
7	M	16	3.4	62.4	33.3	0	0.9
8	M	9	0.5	60.5	37	0.5	1.5
9	M	5	3.5	57.9	38.2	0	0.4
10	F	6	1.8	47.6	49.8	0.3	0.5
Median (min, max)			2.7 (0.5, 6.1)*	54.3 (41.6, 69.3)	41.8 (24.3, 55.2)	0.2 (0, 0.7)	0.7 (0, 3.5)
11	M	5	5.3	49.3	43.9	0	1.5
12	F	13	50	23.9	25.7	0	0.4
13	F	6	12.6	48	37.4	0	2
14	M	17	5.8	38.7	55.5	0	0
15	M	18	16.7	43.4	30.8	6.3	2.8
16	M	16	5.6	30.1	60.6	2.8	0.9
17	F	10	12.8	51.4	34.5	0.7	0.7
18	M	16	26.5	48.1	22.7	0	2.7
Median (min, max)			12.7 (5.3, 50)*	45.7 (23.9, 51.4)	36 (22.7, 60.6)	0 (0, 6.3)	1.2 (0, 2.8)

*$P \leq 0.01$ (Mann Whitney test).

compromised horses was observed (Fig 2, P = 0.084). This trend is in agreement with data from human with chronic airway inflammation who exhibit lower A_{2B}AR expression, suggesting a role for adenosinergic signalling in equine airway disease. Note that A_1AR was not detected in any BAL samples and that A_3AR expression levels were marginal in both groups (not shown).

Induction of IL-6 by the adenosine analogue NECA

Based on these observations, we wanted to determine whether AR activation could trigger the expression of inflammatory cytokines in BAL-cells. Because IL-6 is a major inflammatory cytokine regulated by several stimuli, we decided to investigate whether AR engagement could affect its expression in BAL cells. First, we determined IL-6 baseline expression levels in all samples relative to those of GAPDH. Figure 3a shows expression levels in nonstimulated cells from airway-compromised and from healthy controls. IL-6 mRNA abundance did not differ significantly between both groups. Furthermore, RT-PCR cycle numbers for this cytokine in both groups were close to threshold of detection (cycle 35), implying that IL-6 was minimally transcribed in resting cells from either group.

Subsequently, the cells were treated for 3 h with the nonselective AR agonist NECA and IL-6 induction reassessed (Fig 3b). While cells from healthy controls failed to upregulate this cytokine (1.54-fold, not significant), stimulation of cells from airway-compromised horses resulted in a significant increase of IL-6 transcript (2.51-fold), which was maximal at this time point. This increase in IL-6 expression in NECA-stimulated BAL cells from diseased horses was significantly greater than that of control horses ($P<0.01$). This suggests that BAL-cells from affected animals differentially react to the endogenous nucleoside adenosine.

Differential induction of IL-6 in BAL-recovered cells by AR-subtypes

The A_{2A}AR subtype has been linked with anti-inflammatory functions at several levels and it has been suggested to be a critical part in negative feedback mechanisms for the limitation and termination of inflammatory responses (Ohta and Sitkovsky 2001). Therefore, we wanted to assess the role of this receptor subtype in inflammatory disease in equine airways. More precisely, our aim was to determine whether activation of this AR-subtype could influence adenosinergic proinflammatory signalling in BAL-recovered cells. Therefore, we first tested whether A_{2A}AR activation could influence IL-6 expression. As expected, activation of A_{2A}AR with the selective agonist CGS-21680, at a concentration that has been shown to effectively inhibit LPS-mediated ROS production in equine neutrophils (Sun *et al*. 2007), did not induce IL-6 expression in BAL cells either from airway-compromised or healthy controls (Fig 4a). Further, we evaluated whether A_{2A}AR blockage could influence IL-6 upregulation by NECA, by treating cells with a combination of NECA plus the A_{2A}AR antagonist SCH-58261. Because this is the first study in which equine cells are exposed to SCH-58261 the concentration of 1 µmol/l was extrapolated from binding studies in rat and bovine tissues and functional assays in rabbit and porcine systems (Zocchi *et al*. 1996). While SCH-58261 did not affect IL-6 expression, the combined treatment of NECA and SCH-58261 resulted in a moderate but significant increase of IL-6 expression in healthy controls (median 5.28; s.d. = 1.96), when compared to NECA alone. Similarly, A_{2A}AR blockage in airway-affected horses resulted in prominent increases in IL-6 induction by NECA, reaching up to 29-fold upregulation (median = 13.04; s.d. = 7.84). In addition, IL-6 upregulation by combined NECA and SCH-58261 treatment was significantly different between both groups (Fig 4a). Altogether, this data shows that activation of A_{2A}AR in BAL-recovered cells can modulate proinflammatory signalling by other ARs, particularly in airway-affected horses.

Finally, we examined whether this differential IL-6 induction by AR activation could be associated with the changes in BAL cytology. Indeed, we detected a correlation between neutrophil count and IL-6 induction by NECA plus SCH-58261 for all 18 horses included in this study (Fig 4b), suggesting a causal relationship between the neutrophil number and AR-dependent

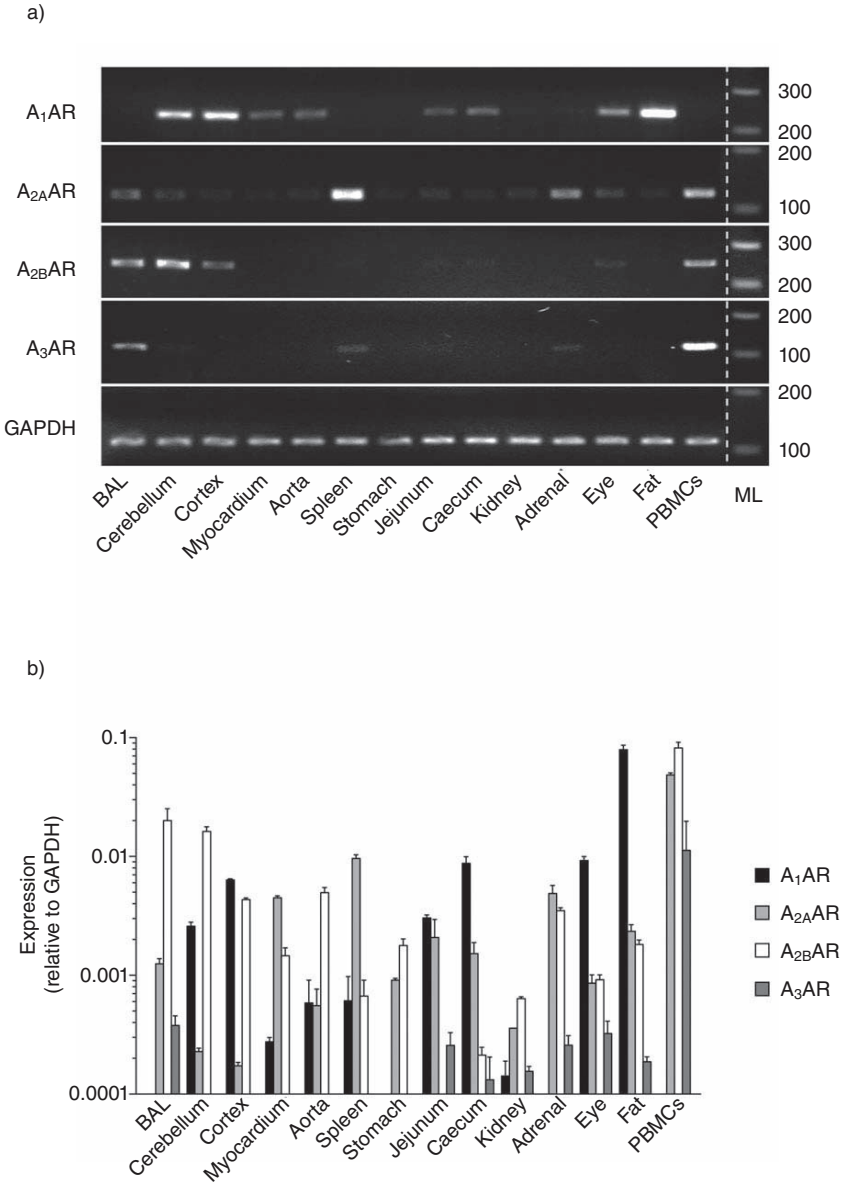

Fig 1: Expression profiles of ARs in tissue samples from a single healthy horse, assessed by RT-PCR. a) Finalised reactions (cycle 45) were loaded in an agarose gel for side by side for comparison. GAPDH reactions are included as a loading control. ML: DNA molecular ladder, sizes are given in base pairs. b) Quantitative analysis. The values are relative to GAPDH and indicate mean and standard deviation of 3 independent measurements.

IL-6 induction. A similar correlation was observed with IL-6 induction by NECA (not shown).

Discussion

In this study we first characterised the expression of adenosine receptors in BAL samples, as compared to other tissues. Besides the tissue-specific variations that hint towards divergent adenosinergic pathways in different organs and systems, whose interpretation exceeds the scope of this study, we observed a remarkable similarity between the AR profiles of BAL-cells and PBMCs. A_{2B}AR was predominantly expressed in both of these cell types, followed by A_{2A}AR and lower A_3AR levels. This analogy and the fact that neutrophils express primarily A_3AR (Varani

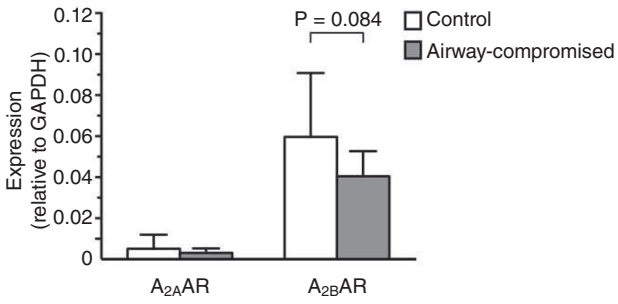

*Fig 2: AR expression in BAL-samples from airway-compromised and control horses, by RT-PCR. Expression values are relative to GAPDH and indicate mean and standard deviation of 3 independent measurements (*t *test with Welch correction).*

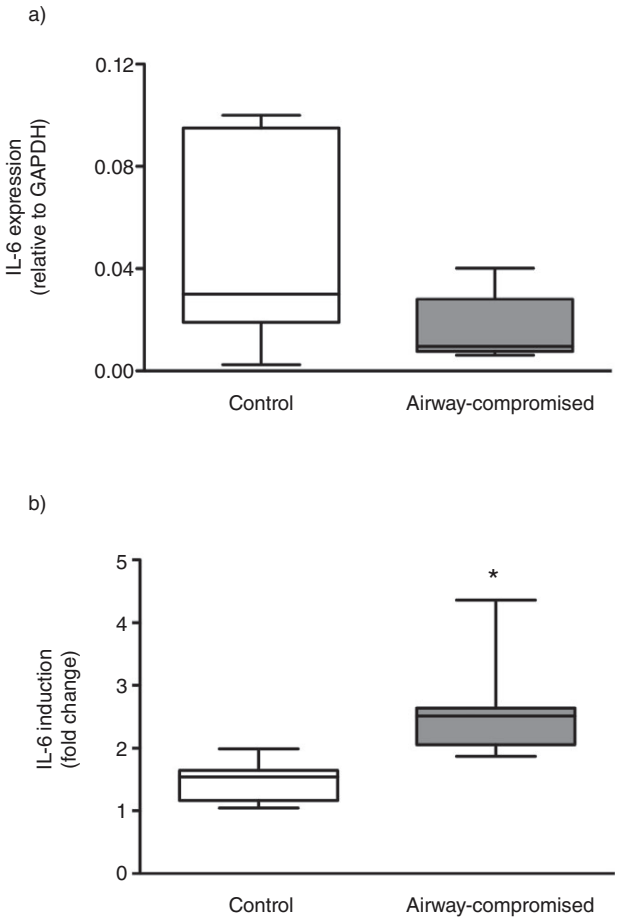

*Fig 3: a) Expression of IL-6 in resting, nonstimulated BAL cells. cDNA was obtained from each horse and IL-6 abundance assessed by RT-PCR. Expression values are relative to GAPDH. b) Induction of IL-6 by NECA in BAL cells. Values for each reaction were normalised against GAPDH and fold changes calculated in relation to basal values for each horse (resting cells). Whiskers represent 10 and 90 percentiles. *: P≤0.01 vs. basal values and between groups (Mann-Whitney U test).*

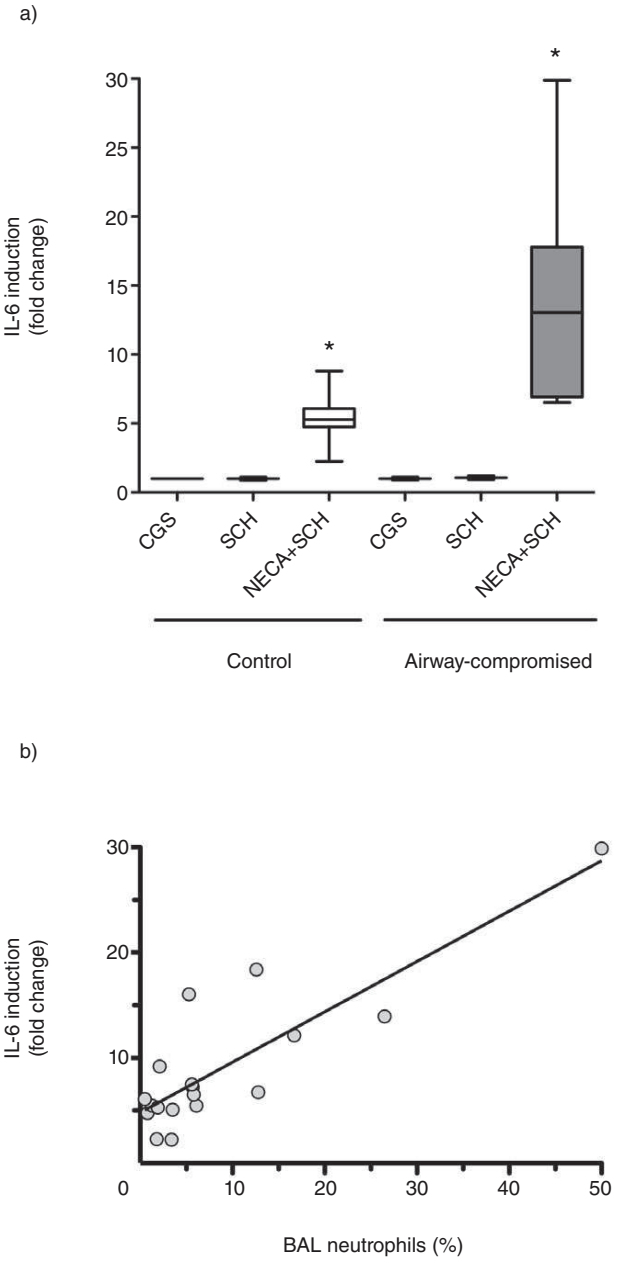

*Fig 4: a) Effect of A_{2A}AR blockage on NECA-mediated IL-6 induction. Fold changes are calculated in relation to basal values for each horse (resting cells). Whiskers represent 10 and 90 percentiles. *: P≤0.01 vs. basal values and between groups (Mann-Whitney U test). b) Correlation between neutrophil percentage and IL-6 induction by NECA plus SCH-58261 for all 18 BAL samples. Spearman correlation coefficient r = 0.66, P≤0.01.*

et al. 2009) insinuate that lymphocytes and macrophages are the predominant AR-expressing cells in BAL (together they represent over 90% of BAL cells). Furthermore, these observations suggest that adenosine could play an immunomodulatory function in the airways in the horse.

In line with this hypothesis, we show enhanced reactivity of BAL-cells from airway-compromised horses upon *ex vivo* AR stimulation, thereby establishing a link between adenosine signalling and airway inflammation in this species. RT-PCR analysis revealed increased expression of IL-6 upon nonselective AR activation with the adenosine analogue NECA in cells from airway-affected horses, while this response was absent in control animals. This amplified response to adenosine could be explained by adaptive responses of airway/BAL-cells to the inflammatory milieu. Long-term *in vitro* exposure studies have indeed shown desensitisation of ARs by adenosine in target cells (Fredholm *et al*. 2001). In COPD patients, for example, elevated concentrations of adenosine and other inflammatory mediators in the peripheral lung tissue correlated with downregulation of the A_{2B}AR and with increased densities of the A_{2A} and A_3AR (Varani *et al*. 2006). In line with that report we detected a trend towards a decrease in A_{2B}AR in airway-affected horses, which was not significant (possibly because of the heterogeneity in AR expression, particularly among healthy horses). A systematic analysis of AR expression by cell type in a larger sample group would be necessary to confirm of this trend.

The exaggerated response of airway-affected horses can also be explained by the alterations in their BAL cytology. In fact, we identified a correlation between percentage of neutrophils in BAL and cell reactivity to adenosinergic proinflammatory signalling. This observation, however, does not allow to establish whether IL-6

upregulation depends on neutrophil count, or, on the contrary, whether higher neutrophils in BAL are the result of increased proinflammatory AR signalling with elevated levels of IL-6 and probably of other neutrophil chemotactic factors.

IL-6 is a multifunctional cytokine that has been associated with inflammation and the development of immune responses. Although initially considered a surrogate marker of inflammation (similar to IL-1β and TNF-α) it is now clear that IL-6 can also influence the effector functions of various $CD4^+$ T cell subsets. IL-6 inhibits Th1 differentiation and promotes Th2 differentiation during early $CD4^+$ T cell activation, and also induces the differentiation of Th17 effector cells in the presence of TGF-β (Mangan *et al*. 2006). IL-6 also inhibits regulatory T cell development, most likely by suppressing Foxp3 expression (Dominitzki *et al*. 2007). Its role in promoting Th2 and Th17 differentiation, along with inhibiting regulatory T cell activity, suggests that IL-6 might play a role in the onset and/or progression of diseases associated with these types of immune responses, including RAO. Interestingly, engagement of proinflammatory ARs has been linked to increased release of IL-6 from epithelial cells, astrocytes and fibroblasts (Sitaraman *et al*. 2001; Zhong *et al*. 2005). In addition, a recent study using Calu-3 human epithelial cells demonstrated that extracellular adenosine induces a robust release of IL-6, establishing a connection between adenosine and neutrophil infiltration in the airway lumen (Sun *et al*. 2008b).

Activation of A_{2A}AR has been associated to anti-inflammatory effects, including modulation of neutrophil and mast cell activation and degranulation, downregulation of oxidative species and adhesion molecules and inhibition of cytokine release (Lappas *et al*. 2005). In the lung, activation of the A_{2A}AR was linked to downregulation of inflammation in models of allergic asthma (Koshiba *et al*. 1999; Fozard *et al*. 2002). A_{2A}AR activation also attenuated IL-6 levels associated with inflammatory lung injury in a rat model for cardiopulmonary bypass (Mohsenin *et al*. 2007) and recent evidence indicates this AR can modulate inflammatory signals in the horse. For example, activation of the equine A_{2A}AR in a heterologous system was shown to inhibit the proinflammatory factor NF-κB (Sun *et al*. 2008a). Also, engagement of A_{2A}AR on LPS-activated equine peripheral blood monocytes led to a decrease in expression of COX-2 and TNF-α and upregulation of IL-8 and IL-10, implying a role of this receptor in the modulation of inflammatory signals (Sun *et al*. 2010). Here, we investigated for the first time the role of A_{2A}AR in the airways. Selective blockage of the A_{2A}AR in BAL-cells with SCH-58261 allowed us to distinguish between the effect of this receptor subtype and the composite effect of all ARs by NECA, thereby revealing the existence of functional A_{2A}AR in equine airways. Because activation of nonA_{2A}ARs in BAL cells with NECA plus SCH-58261 resulted in exaggerated IL-6 transcription, it can be reasoned that A_{2A}AR downregulates the induction of the proinflammatory IL-6 induction by the other ARs expressed in these cells (mainly A_{2B}AR). This effect on the expression of this proinflammatory cytokine supports the idea of selective pharmacological activation of A_{2A}AR as a therapeutic principle in airway inflammation in the horse.

In summary, this study shows that IL-6 is rapidly induced in BAL-cells of airway-compromised horses in response to adenosine exposure, probably through A_{2B}AR activation and that this effect can be modulated by A_{2A}AR. Further analysis of the effect of A_{2A}AR activation on other inflammatory stimuli will be required for a more complete assessment of the therapeutic potential of selective AR activation in the horse.

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Authors' declaration of interests

No conflicts of interest have been declared.

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Manufacturers' addresses

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[7]Ficoll-Paque PLUS, GE Healthcare Biosciences, Uppsala Sweden.
[8]Bio-Rad, Richmond, California, USA.
[9]SPSS Inc., Chicago, Illinois, USA.

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Supporting information

Additional Supporting Information may be found in the online version of this article: www.wileyonlinelibrary.com/journal/evj

Fig S1. Primer specificity. RT-PCR reactions were performed with the indicated primers and BAL or cerebellum cDNA. The reactions yielded single bands at the expected sizes and no unspecific secondary bands are observed. A reaction for GAPDH amplification without DNA is shown (-). ML: DNA molecular ladder, sizes are given in base pairs.

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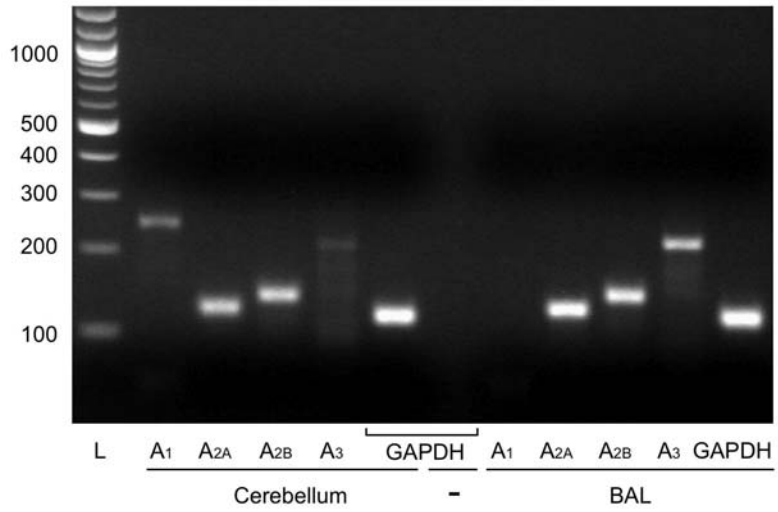

Supplementary Figure

Supplementary figure. Primer specificity. RT-PCR reactions were performed with the indicated primers and BAL or cerebellum cDNA. The reactions yielded single bands at the expected sizes and no unspecific secondary bands are observed. A reaction for GAPDH amplification without DNA is shown (-). ML: DNA molecular ladder, sizes are given in base pairs.
175x221mm (300 x 300 DPI)

8 Summary and conclusions

Treatment of inflammatory diseases today is targeted towards interfering with the synthesis of mediators driving inflammation. This concept is based on the 'traditional' view that considers that once the inflammatory response has neutralized an injurious stimulus, inflammation *somehow* fades away, possibly from inflammatory mediator catabolism. Non-steroidal anti-inflammatory agents, steroids and antihistamines were developed on this basis. In recent years, however, it has become evident that the resolution of inflammation is a highly coordinated and active process controlled by endogenous 'pro-resolving' factors. These factors switch off leukocyte trafficking to the inflamed site, reverse vasodilation and vascular permeability, and bring about the safe disposal of inflammatory leukocytes, exudate and fibrin, thereby leading to the restoration of the inflamed tissue to its prior physiological function. As a consequence, successful resolution will limit excessive tissue injury and give little opportunity for the development of chronic, immune-mediated inflammation. The anti-inflammatory and tissue protective effects of adenosine seem to indicate that this is indeed an inflammatory pro-resolving factor. However, there is also a negative side of this molecule, and the intracellular mechanisms dictating towards an inflammatory shutdown or a perpetuation of the inflammatory state are only starting to become understood.

In the first part of this work we employed a transcriptomics approach for the identification and characterization of downstream effector mechanisms of ARs. We reasoned that by employing selective AR activation in combination with a genome-wide approach we would be able to identify molecular players that contribute to the shutdown or perpetuation of inflammation. Through this approach we were able to establish a novel functional link between adenosine signalling and the nuclear orphan receptor 4A family of transcription factors and we show that selective activation of the A_{2A}AR can negatively regulate both the induction and the activity of these factors. The most exciting finding of this study was the fact that the concomitant treatment with the A_{2A}AR antagonist SCH-58261 significantly amplified NECA's effect on NR4A2 and NR4A3 induction; given the pleiotropic roles of this family of transcription factors, adenosine's effect on these could have broad implications both in physiological states and disease. We further characterized the intracellular signalling pathways associated with the upregulation of NR4A2 and NR4A3 and established that PKC and MEK kinases participate in the induction of these factors. Interestingly, some studies have also shown the involvement of ERKs in adenosine signalling (Feoktistov et al., 1999) but downstream targets of ERK were not known. The data presented in this manuscript show that that activation of ERK1/2 kinases downstream of PKC mediates NR4As induction by AR, at least

partially (at least one additional intracellular signalling pathway must be involved in AR-dependent induction of NR4As, because blockage of PKC and a complete inhibition of ERK phosphorylation resulted only in about a 50% decrease NR4A2 and NR4A3). Most strikingly, the A_{2A}AR modulatory effect on the ERK1/2-NR4A signalling axis is not limited to adenosinergic signalling, but also to PMA: A_{2A}AR engagement modulated PMA-mediated ERK1/2 phosphorylation, limited NR4A2 and NR4A3 induction and their activity as assessed by a luciferase reporter assay. Taken together, these results establish a novel effector signalling axis downstream of adenosine, and suggest NR4A antagonism as an inflammatory shutdown mechanism triggered by A_{2A}AR activation in mast cells.

In the second part of this study we explored the potential of AR-based therapies in veterinary medicine, centering our focus on RAO, a common airway disease in the horse. Our approach was to analyse the presence of a functional adenosine signalling axis in the equine airways. Therefore, the first step was to determine: a) whether adenosine can be detected in equine airways; b) if a significant difference in the concentration of this nucleoside between BAL samples from healthy and airway compromised individuals can be established; c) whether adenosine concentrations correlate with the other major criteria for diagnosis of RAO, namely clinical findings and neutrophil count in BAL. In chapter 6 we addressed this issue by measuring adenosine concentrations in BAL samples from 11 horses with non-infectious lower-airway inflammation and 14 healthy controls with a specific spectrometry-based assay. Not only was adenosine detected in all samples examined, but also adenosine concentration was significantly higher in RAO-affected horses, suggesting that this nucleoside could play a significant role in lower airway inflammation in this species. Moreover, when plotted against the clinical score, we were able to detect a positive correlation between adenosine concentration and the score, indicating a relationship between this nucleoside and the presence of clinical signs indicative of an inflammatory process in the airways. However, adenosine concentration did not directly correlate to neutrophil count. This is the first report showing the presence of adenosine in the equine airways and laid the basis for our subsequent studies.

To explore the idea that adenosine may contribute to lower airway inflammation in the equine airways in more depth, we analysed both the expression of ARs in BAL cells and the reactivity of these cells to AR ligands. The first major finding of the study in chapter 7 is the fact that BAL cells express predominantly A_{2B}AR, with lower levels of A_{2A}AR. Notably, this pattern is similar to that of PBMCs and different from other tissues tested, suggesting that lymphocytes and macrophages are the predominant AR-expressing cells in BAL (together they represent over 90% of BAL cells) and that adenosine may modulate the activities of these cells in the equine airways. The second major message from this study is that BAL-cells from airway-compromised horses show enhanced reactivity to AR ligands, when measuring

induction of IL-6 by RT-PCR. Remarkably, BAL-cells cells from airway compromised horses significantly upregulated this cytokine, while this response could no be detected in clinically sound horses. To further evaluate the potential role of the A_{2A}AR in modulating inflammatory signalling in the equine airways BAL cells were concomitantly treated with NECA and the A_{2A}AR antagonist SCH-58261. We reasoned that if a functional A_{2A}AR were present, blockage of this receptor subtype would result in a further elevation of IL-6 expression. In fact, we observed that SCH-58261 enhanced IL-6 mRNA induction by NECA both in airway compromised and in control horses, but that much higher IL-6 levels were reached in airway-compromised horses. These findings could be explained either by adaptive responses of airway/BAL-cells to the inflammatory milieu or by an altered cytological profile in samples for airway-compromised horses. As a matter of fact, we established a correlation between the percentage of neutrophils present in BAL samples and the reactivity to adenosinergic proinflammatory signalling. However, this observation does not allow us to conclude whether IL-6 upregulation depends on neutrophil count, or, on the contrary, whether higher neutrophils in BAL are the result of increased proinflammatory AR signalling with elevated levels of IL-6 and probably of other neutrophil chemotactic factors. These findings confirm the presence of an adenosine/IL-6 inflammatory axis, which is overrepresented in the bronchoalveolar milieu of airway-compromised horses and that even though A_{2B}AR is the predominant AR subtype expressed in equine BAL cells, A_{2A}AR seems to be able to modulate proinflammatory IL-6 signalling by adenosine. In summary, our observations in BAL samples strongly support the notion that adenosine contributes to inflammation in the airways of horses with RAO and constitutes the basis for more specific research of AR-based therapies for equine RAO and possibly for other chronic airway diseases in the horse.

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